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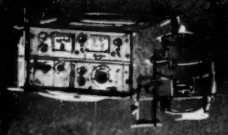

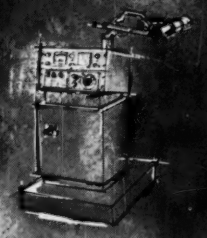

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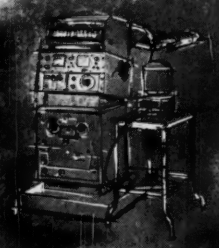

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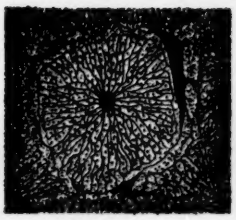

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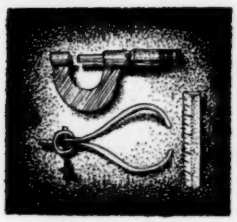

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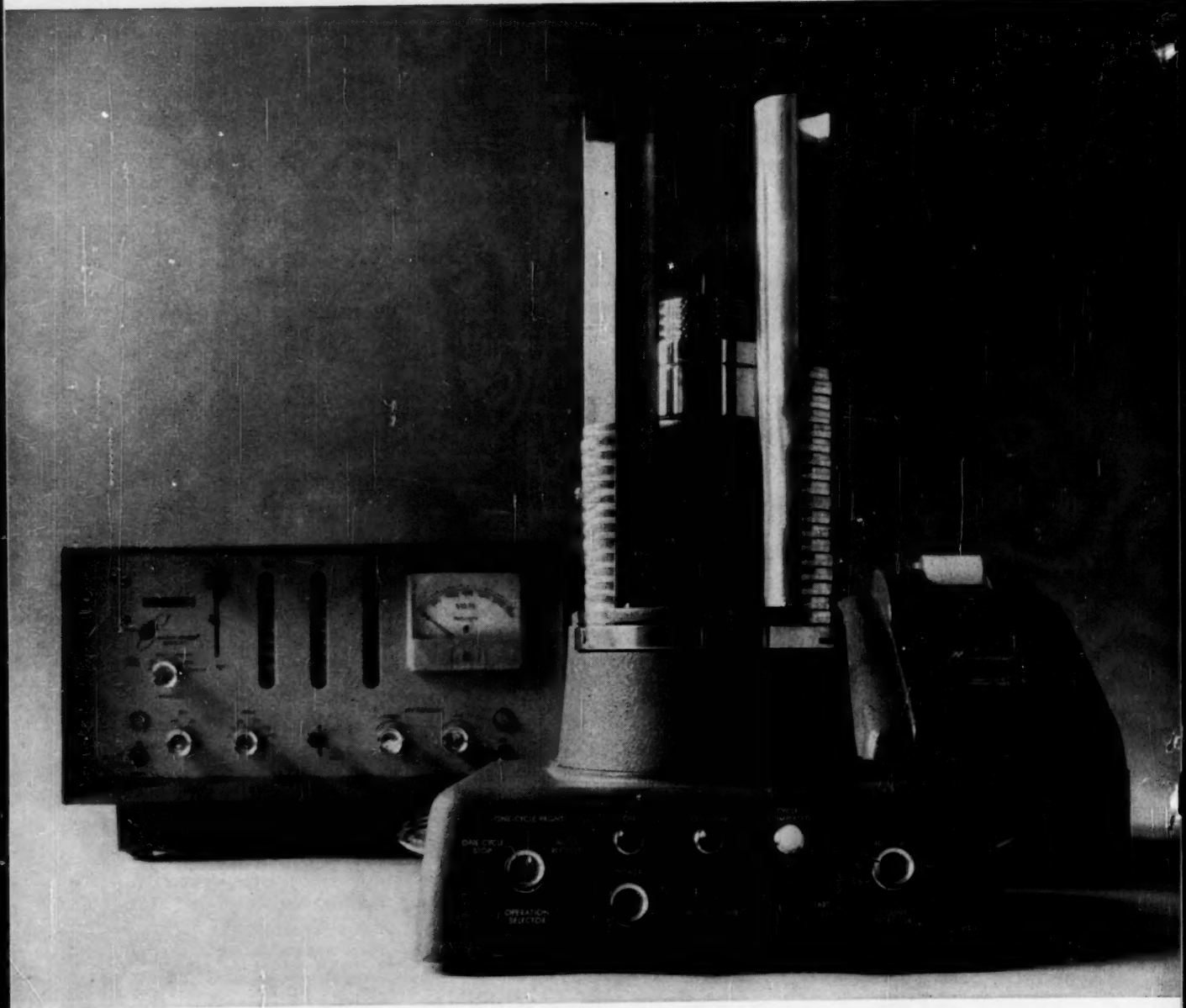

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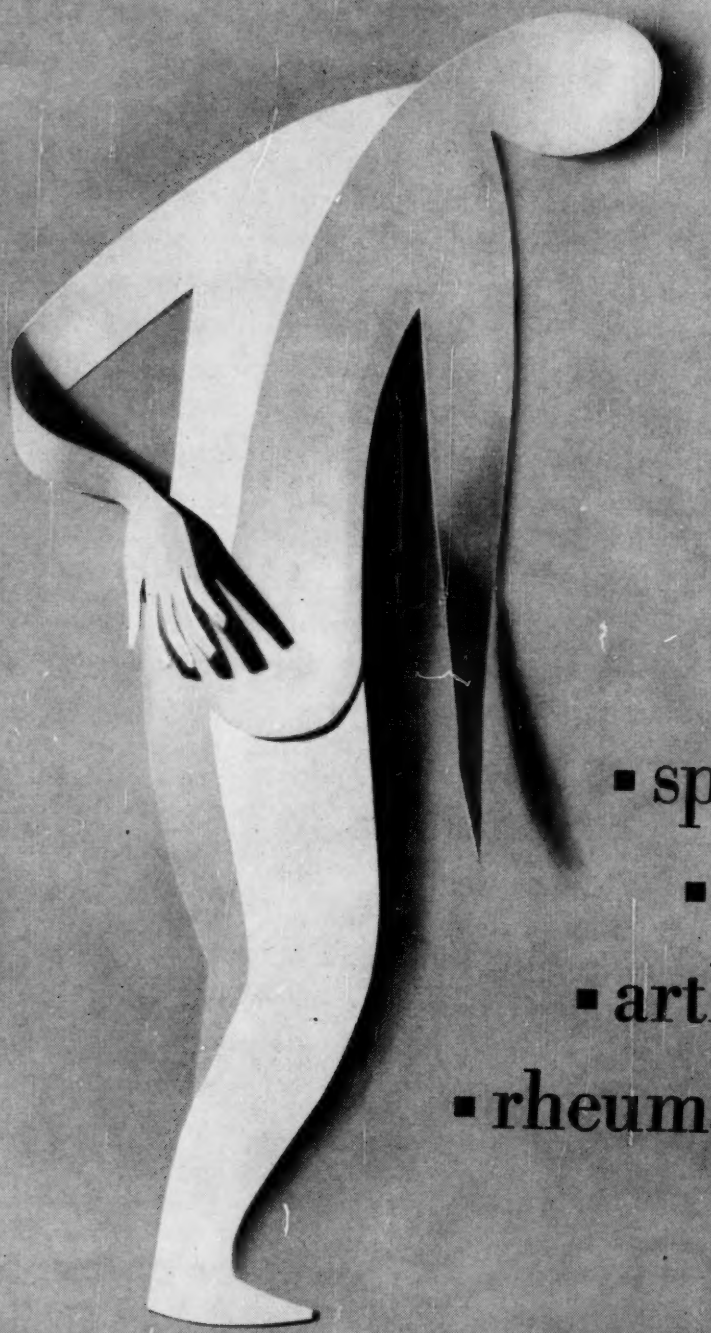

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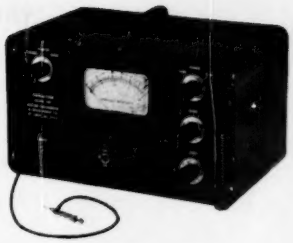

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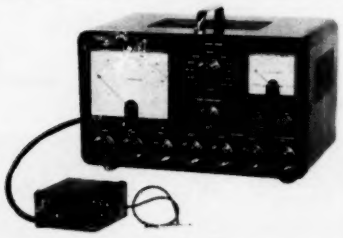

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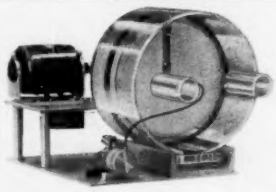

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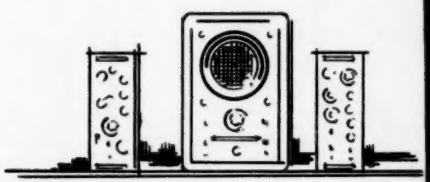

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RELATION OF HEMOGLOBIN TO THE RED CELL MEMBRANE * †

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(Submitted for publication June 29, 1959; accepted July 31, 1959)

Despite the accumulation of biochemical and physiological knowledge of hemoglobin, little is certain about its physical state in the native red blood cell. Perutz and Mitchison (1) indicate that the pigment is almost surely not in the crystalline state except for sickle-hemoglobin in the reduced form. A separate aspect of the problem is the relation of hemoglobin to the cell membrane. The prevailing view is that of the respiratory physiologists who hold that the exchange of blood gases involves no chemical event in the membrane (2). Accordingly, hemoglobin is excluded from it. Drabkin has estimated that not more than about 2 per cent of the total pigment could be "anchored at the surface of the red cell" (3), while Hoffman has recently concluded emphatically that the notion that there may be structurally fixed hemoglobin must be abandoned (4). Yet, many investigators have been struck by the difficulty encountered in trying to free red cell stromata entirely from hemoglobin. For example, Zittle, Della Monica and Custer, in preparing red cell acetylcholinesterase, noted the stromata contained "considerable hemoglobin in spite of the long period of washing" (5). Ponder, in a recent review (6), favors the idea that some of the pigment is associated with the membrane. His view is based in part on microscopic observations that could bear other interpretations and afford no quantitative data, and in part on the finding that "ghosts" may contain a greater concentration of hemoglobin than the surrounding lytic solution, "provided that the amount of water used for hemolyzing the cells is relatively small." Unfortunately, with only small amounts of water lysis is likely to be incomplete, and it is accordingly improbable that there is free diffusion of pigment between cells and medium. Unless the condition of free diffusion is satisfied, differences of concentration of intracellular and extracellular hemoglobin cannot be regarded as evidence of binding between hemoglobin and stroma rather than as a reflection of membrane phenomena such as permeability.

Two main difficulties have stood in the way of approaching the issue more directly. First, there has not been available a satisfactory method of measuring amounts of hemoglobin in membranes directly, on account of their insolubility. A few investigators have measured iron, but the amounts found have been variable and inconstant and, furthermore, not certainly associated with hemoglobin itself. Secondly, although various ways of preparing ghosts have been devised, yet it is rarely clear to what extent the processing may have resulted in loss of stromal substance. Thus, it has not been common practice to measure any characteristic membrane constituent, *e.g.*, phospholipid, to see whether the quantity found approximates that of the native cell. The production of "hemoglobin-free ghosts" affords no warranty as to the degree of preservation of any of the natural components of the intact membrane and, indeed, microscopists have come to speak of "attenuated" or "thin" ghosts (6). Experiments of Lovelock illustrate the delicate constitution and fragile structure of red cells, for even repeated washings with saline at 37° C. may result in appreciable losses of lipid and protein although hemolysis is negligible (7).

For estimating residual hemoglobin attached to membranes, a simple direct method has recently been devised which involves dissolving weighed amounts of lyophilized membranes in 40 per cent urea and measuring photometrically the concentration of cyanmethemoglobin (8). It could then be shown for one way of processing cells that substantial and rather constant amounts of hemo-

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globin are demonstrable in membranes even after thoroughgoing lysis and repeated washing with water. At the same time reproducible and regular quantitites of nonhemoglobin nitrogen and of lipid phosphorus were found and it was possible to describe the preparation of red cell membranes in terms of quantitative chemical analyses (8). The present work represents an amplification and extension of the observations. The applicability of the methods to cells of animals has been examined and various methods of preparing membranes compared. It will be shown that it is extremely difficult to reduce the amount of hemoglobin attached to membranes beyond a certain minimum without at the same time removing either lipid phosphorus or nonhemoglobin nitrogen or both. The data support the idea that in the native cell about 3 per cent of the total blood pigment is bound to the membrane. Since the term "ghost" has come to be applied to almost any sort of preparation of red cells which have been somehow partially hemolyzed, it seems best for present purposes to avoid its use and to speak definitively of "membrane," meaning the insoluble phase of the cell as separated *in vitro*.

METHODS

In an extension of studies of species differences in erythrocyte phosphatides (9), variations in yields of phosphorus were traced to variations in the anticoagulant used, and a method of preparing membranes was therefore developed which overcame this difficulty. In this method, called here for convenience "standard," blood is taken into acid citrate dextrose (ACD), known from blood bank experience to be an excellent preservative. (ACD = 0.4 per cent citric acid, 1.32 per cent sodium citrate, 1.47 per cent dextrose; one part to four parts whole blood.) This and all other solutions were brought to 4° C. before use. Centrifugations were carried out in the high capacity-angle head of the International Refrigerated Centrifuge, Model PR2, at 4° C. and 10,000 rpm (11,000 × G). Samples of human adult blood were fresh or banked as noted. Human fetal blood and the specimens of animal blood were not more than a few hours old when processed. After separation of plasma, the cells were washed three times in cold saline to remove the buffy coat of white cells as well as the remaining plasma and also to bring them to 4° C. before further processing. Lysis was then carried out by repeated exposure to 10 volumes of cold water, the insoluble residue each time being deposited by high speed centrifugation. Other methods of preparation have been examined and the materials obtained have been compared in terms of certain chemical and morphological data. Measurements of lipid phosphorus in the intact red cells were made by extraction into chloroform-methanol and washing with large volumes of water, according to the procedure devised by Folch and co-workers for wet tissues (10).

The harvests of wet membranes, suspended in a minimum of fluid, were lyophilized, and portions then analyzed for nitrogen and lipid phosphorus as previously described (9). That part of dry weight representing hemoglobin was estimated by dissolving 10.0 mg. of lyophilized membranes in 10.0 ml. 40 per cent urea containing standard cyanide-ferricyanide [0.1 per cent $NaHCO_3$, 0.005 per cent KCN, 0.02 per cent K_3 Fe $(CN)_6$] and measuring the concentration of cyanmethemoglobin in a photometer. With occasional shaking a clear solution was usually obtained in an hour or two. Persistent turbidity was encountered rarely, and then only when the method of preparation under trial had involved exposure of the membranes to materials of a kind that might well have produced considerable denaturation of protein. In 20 per cent urea, solution took place more slowly; the results were otherwise no different. It has been shown that strong solutions of urea will not interfere with the absorption spectra of hemoglobin and its derivatives (11). However, it became necessary to look for a spectral disturbance resulting from prior lyophilization. A fresh solution of human hemoglobin was prepared from the supernatant after lysis of cells with water and an aliquot was lyophilized. Using the Beckman DU spectrophotometer, absorption spectra were then plotted for the following: *a*) cyanmethemoglobin standard, *b*) lyophilized hemoglobin dissolved in 40 per cent urea containing cyanide-ferricyanide, *c*) original watery predecessor of *b* after conversion to cyanmethemoglobin, *d*) lyophilized membranes dissolved in urea-cyanide-ferricyanide. The curves obtained were all superimposable and characteristic of cyanmethemoglobin. It appeared, therefore, that lyophilization did not interfere with conversion to cyanmethemoglobin, and further, that the method devised for measuring hemoglobin in membranes was a valid one. Nonhemoglobin nitrogen was estimated as the difference between total nitrogen and hemoglobin nitrogen, calculated as 16 per cent of the pigment.

RESULTS

With the standard method of preparing membranes by repeated exposure to 10 volumes of water at 4° C., the supernatant was usually virtually free of pigment after four times, and the membranes deposited were distinctly reddish. The number of applications of water was increased in many experiments, but further loss of hemoglobin from the membranes was negligible. For instance, in one experiment, a portion of the membranes was washed 12 times in water and during both the eleventh and twelfth washings allowed to stand in the water overnight. The amount of hemo-

TABLE I

Average composition of lyophilized standard membranes prepared after repeated lysis in water at 4° C.

Sources of cells	Hemoglobin	Total nitrogen	Phosphatide*
	mg./100 mg. dry weight		
Human adult (24)†	54	13	10
Human fetal (4)	58	14	11
Rabbit (1)	58	13	15
Guinea pig (1)	56	14	14
Ox (2)	48	11	16

* Calculated as 25 × lipid phosphorus.

† Figures in parentheses represent numbers of individuals. The total yield of dry material was generally just under 2 per cent of the wet weight. One ml. packed cells yielded about 18 mg. dry weight. Standard deviation for human adult = 3 per cent.

globin in all of the washings and in the membranes was measured and it was found that the further loss of "attached" hemoglobin after the fourth wash was only about 5 per cent. The routine was therefore adopted of washing four times with water or, if then the supernatant was still pink, once or twice more. Membranes prepared in this way occupied a packed wet volume about 50 to 60 per cent of that of the intact cells. When examined with the ordinary light microscope, they appeared as disks. This impression was confirmed by phase contrast microscopy (Figure 1). Furthermore, the degree of completion of lysis could be appreciated microscopically, since intact red cells are much more densely pigmented than those that have been lysed. Only very few or no intact red cells were observed after the standard method of lysis. The membrane composition is given in Table I. It can be seen that the amount of hemoglobin found is in the neighborhood of 50 per cent of the total dry weight. The range of values extended from 48 to 58 per cent. This corresponds to about 3 per cent of the total hemoglobin of the intact cell. With human blood banked for one to four weeks, the figure was somewhat lower, usually just below 50 per cent. Included among the human adults were cases of acute and chronic leukemia, idiopathic thrombocytopenic purpura, acquired hemolytic anemia, as well as nonhematological disorders such as cardiac failure. The amounts of hemoglobin found did not vary significantly with anemia or reticulocytosis.

Since the amount of phospholipid nitrogen is so small that it can be neglected, the total nitrogen

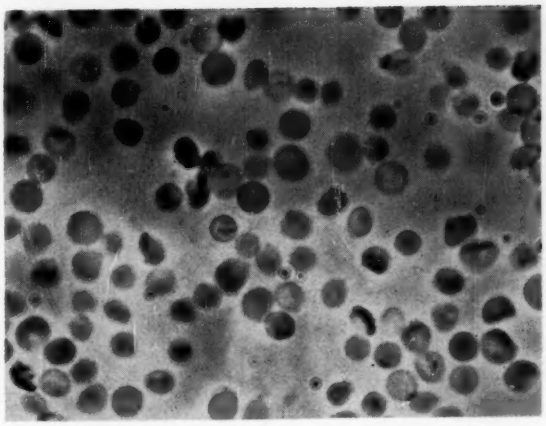

FIG. 1. PHASE CONTRAST PHOTOMICROGRAPH OF "STANDARD" MEMBRANES SUSPENDED IN WATER

Varying degrees of lightness and darkness are determined by optical properties such as depth, and do not signify variations in content of hemoglobin.

may be assumed to represent in effect the contributions of hemoglobin and nonhemoglobin peptide or protein ("stromatin"). The latter, then appears to make up about half as much of the dry weight as does hemoglobin.

The amounts of lipid phosphorus recovered varied somewhat with the species of animal. For human cells, several experiments were carried out to compare the amounts of lipid phosphorus in standard membranes to the amounts in their native wet precursors. Fresh wet red cells subjected to extraction with chloroform-methanol [Folch and associates (10)] yielded 65 to 70 μg. lipid phosphorus per ml. Equivalent amounts of the same samples were processed in the standard way and lyophilized, and the yield of lipid phosphorus found to be 58 to 66 μg. per ml. wet. The loss of phospholipid was thus not more than 10 per cent. With blood that had been banked, losses of phosphorus during processing were occasionally greater, once as much as 30 per cent. It appeared, therefore, that the method employed conserved most of the phospholipid and at the same time yielded regular amounts of packed, wet membranes which had a fairly uniform microscopic appearance as intact disks with little fragmentation, and which gave reproducible analytical values for nitrogen, phosphorus and hemoglobin. It now became of interest to see how these results might be influenced by alteration of the experimental conditions.

TABLE II

Average yield and composition of membranes: Anticoagulants ACD and oxalate compared*

	Lipid phosphorus		
	Native cells	Membranes	Total solids
	μg./ml. wet native cells		*mg./ml. wet native cells*
ACD	65	66	18
Oxalate	70	45	6

* Acid citrate dextrose.

In the first step of the processing, *i.e.*, prevention of coagulation, the use of 1.4 per cent potassium oxalate instead of ACD resulted in a considerable diminution of membrane substance during the subsequent lysis. This is illustrated in Table II, where it can be seen that much of the membrane solids, including phospholipids, was lost when oxalated blood was processed. Also, on microscopic examination, fragmentation of the membranes into fine particles was seen after the third application of water. A systematic study of other anticoagulants was not made, nor were efforts directed toward finding substitutes for ACD. A few trials of heparin and of defibrination revealed membrane injury like that observed with oxalate, since early fragmentation was observed microscopically when lysis was undertaken.

The use of ACD appeared to be a much more important factor in obtaining reproducible results than did the control of temperature, at least for the range 4° to 18° C. However, it was observed that bovine cells treated at 18° C. rapidly became brownish in color, probably from methemoglobin formation, and on this account as well as on the general desirability of maintaining low temperature in handling proteins, all processing was carried out routinely in the cold.

Attention was next directed to the effect of varying the composition of the fluid used for lysis of blood drawn into ACD. Instead of water, hypotonic solutions at acid, alkaline and neutral pH were employed. At pH > 8, lysis was associated with micrscopic fragmentation or thinning of membranes and grossly apparent loss of substance, as for instance with 0.015 M K_2HPO_4, pH 8.5, and the hemoglobin content was reduced to the neighborhood of 25 per cent of dry weight. On the other hand, with 0.015 M KH_2PO_4 or NaH_2PO_4, pH 4.5 to 4.6, membranes were obtained that closely resembled the "standard" ones in appearance and chemical composition. Because of Parpart's report of having obtained hemoglobin-free membranes with carbonic acid (12), particular attention was given to the effect of this substance. When lysis was carried out with water saturated with CO_2, pH 3.8 to 4.0, floating particles (lipid?) were encountered after the third or fourth application. At this time also most of the sedimented residue appeared amorphous and fibrous microscopically, though it still contained some 25 per cent hemoglobin as dry weight. Entirely similar results were obtained with water half-saturated and quarter-saturated with CO_2 and with 0.01 M citric acid, pH 2.7, and citrate-phosphate, pH 4.2. It was concluded that the optimum pH range for processing membranes was somewhere in the range 4.5 to 8.5 and that the use of carbonic acid had best be avoided.

A comparison was made of lysis of cells by water, 0.1 per cent NaCl, 0.3 per cent NaCl, and by 1, 2.5 and 5 per cent glycerol in water. All of these agents produced microscopically intact membranes giving a similar appearance and yielding comparable quantities of total dry weight solids; the amounts of hemoglobin as dry weight found were: for water 58 per cent; for 0.1 per cent NaCl, 45 per cent; for 0.3 per cent NaCl, 45 per cent; all glycerol solutions, 40 per cent. It seemed probable, therefore, that a number of solutes might be found which in hypotonic solution at neutral pH could serve much like water for lysis of cells and preparation of membranes. However, when in the course of lysis a change was made in the lytic agent, then loss of membrane substance was often seen. This is illustrated in Table III. Here a method of preparing membranes with progressively decreasing concentrations of NaCl, sometimes referred to as "osmotic shock" (13), is examined. It may be noted that the treatment resulted in a very considerable loss of total substance, representing hemoglobin, nonhemoglobin protein, as well as phospholipid. It was concluded that although certain hypotonic solutions, if used alone, might perhaps be substituted for water as lytic agents, yet a shift from one of these to another during the processing introduces the possibility of denaturation and fragmentation. In any case, the phenomenon of lysis by water or by hypotonic solutions probably involves forces which

TABLE III

Yield and composition of membranes: Water and decreasing NaCl concentrations compared as lytic agents

Lytic agents	Recovered in membranes/ml. wet native cells			
	Total solids	Hemoglobin	Non-hemoglobin peptide	Phosphorus
	mg.	*mg.*	*mg.*	*μg.*
A. Water, 4 washes	18	9	5	70
B. NaCl, 3 washes *1.* 0.45%; *2.* 0.23% *3.* 0.11%	7	3	3	50
C. **B**, then water	5	2	1	35

are obscured in descriptive terms such as "osmotic shock" or "osmotic fragility." It is by no means clear, for instance, to what extent the explosive hemolysis effected by water is determined simply by diffusion.

From the observations detailed above it seemed likely that a small amount of blood pigment is so constantly found in association with the red cell membrane that it might properly be regarded as a membrane constituent. However, among other objections to this idea, it could be said that the hemoglobin is merely trapped within a membrane that has become impermeable to it by, say, denaturation. To meet this argument, fresh preparations of "standard" wet membranes in water were homogenized for 15 minutes in a Virtis homogenizer at 45,000 rpm. This resulted in a very fine fragmentation; few intact membranes could be seen microscopically. The residues were now washed further with 10 volumes of water, in the usual way, three times more. After lyophilization the hemoglobin content was found to be 43 per cent. This figure is, to be sure, lower than the mean (54 per cent), yet considerable enough to refute the idea that trapping is the principal factor accounting for the association of hemoglobin with the membrane.

Standard wet membranes suspended in water exhibited the absorption bands of oxyhemoglobin when examined with the Hartridge reversion spectroscope. It was of interest to see whether conversion of the pigment, at an early stage of the processing, to the derivatives, reduced hemoglobin, carboxyhemoglobin and methemoglobin, might affect the amounts found attached to the membranes. Membranes were prepared *a*) under N_2 with equilibration by bubbling, *b*) after equilibration with CO, *c*) after treatment with nitrite. The amounts of hemoglobin recovered from the membranes as dry weight were: 52 per cent for *a*; 47 per cent for *b*; 43 per cent for *c*; and 49 per cent for control (oxyhemoglobin). It appeared, therefore, that the state of oxidation or oxygenation of hemoglobin did not materially affect its attachment to the membrane.

DISCUSSION

Among the factors that may influence or alter the structure of membranes obtained by any method of hemolysis are: species of animal, kind of anticoagulant, ionic composition of lytic agent, pH, and temperature. There is perhaps nothing astonishing here, since the membrane contains proteins as well as complexes about which little is known, and any of them could be easily denatured. Nonetheless, for those concerned with the chemistry or metabolic behavior or ultrastructure of membranes, it is of importance to delineate as specifically as possible the variables which may condition the composition of the object of study. While it is impossible to define the native membrane properly, yet some knowledge, however imperfect, of its chemistry and architecture can be obtained. But if the results of electron microscopy are to be compared with, say, those of chemical analysis, the methods of preparation had best be uniform. At the same time it is entirely probable that any given method of obtaining membranes, including the one called here, for convenience, "standard," may denature or remove certain constituents. For some purposes, therefore, lysis with hypotonic salt or carbohydrate could well prove superior. The method described above was developed for the principal end of analyzing phosphatides and its further utility remains to be determined. It would be of interest, for example, to know what the enzymatic and metabolic activities of "standard" membranes may be.

The mere association of blood pigment with membrane does not, of course, constitute convincing evidence that the two are somehow bound, and indeed most investigators have regarded the phenomenon as an artifact. On the other hand, the amounts of hemoglobin that have been found during this investigation have been so considerable

(about 50 per cent of the total dry weight) and so regular (range, 48 to 58 per cent) as to cast doubt on the popular idea that the hemoglobin is nothing more than a contaminant. Admittedly, the view that hemoglobin is indeed a constituent of the membrane must be tested further, particularly with attempts to find out more about the nature of the binding, perhaps by isolation and definition of a hemoglobin complex such as a lipoprotein. It may be pointed out that firm binding of a well-characterized soluble protein to the erythrocyte membrane is already known. Acetylcholinesterase travels with the stroma and can be extracted therefrom by non-ionic detergents. This has led to the suggestion that the link between enzyme and membrane may be lipid (5). Moreover, Chargaff, Ziff and Hogg have shown that some phosphatides may react with oxyhemoglobin at neutral pH and even destroy its characteristic spectrum (14). However, since little is known about the forces uniting peptide to lipid in lipoprotein complexes and since some may be dissipated even by removal of water (15), the problem posed by the relation of hemoglobin to the membrane is not likely to be settled easily. The instability of certain lipoprotein complexes offers a possible explanation of so-called "hemoglobin-free ghosts," if hemoglobin is a constituent of the membrane. In any case, however, "hemoglobin-free ghosts" have not usually been analyzed chemically. A well know method of obtaining membranes, by lysis with carbonic acid, is that of Parpart (12), whose preparations contained < 0.01 per cent iron. The species of animal used in the studies is not given, nor are the data that are otherwise supplied recorded in enough detail to permit a judgment as to the degree of resemblance of the final product to the native membrane. With the various methods employed in the present investigation, including that of lysis with carbonic acid, hemoglobin was never lost alone.

It is worth noting that the regular amount of hemoglobin found to be attached to the membrane (3 per cent of the total in the cell) is in the neighborhood of the amount (2 per cent) calculated by Drabkin as unlikely, yet permissible, on the basis of his assumptions as to cell surface and molecular size of hemoglobin (3). But if indeed hemoglobin is a constituent of the membrane, it is at present a matter of speculation as to how it may be laid down and whether it is in equilibrium with the bulk of the cellular pigment.

The problem of the fixation of hemoglobin is only a part of the more general one of membrane constitution. Despite the obvious difficulties, recent development of methods for the analysis of all types of lipids offers promise of progress. For instance, it has been shown that lecithin may be lacking from the red cell membranes of the true ruminants (9) and that the lipid complexes, presumably lipoprotein in nature, of the curious oval red cells of the camel are peculiarly resistant to the disruptive action of alcohol (16). The data at hand are still meager, yet it can scarcely be doubted that any information about the complex structure of a given membrane would be of advantage for physiological studies such as those of ion distribution and permeability.

SUMMARY

The problem of the relation of hemoglobin to the red cell membrane is reviewed. A method is described for preparing membranes and it is shown that they contain reproducible amounts of hemoglobin, nitrogen, phosphorus and total solids. Denaturation and fragmentation of membranes occur readily with variation of the experimental conditions in any stage of the processing and the anticoagulant used is of particular importance. A simple direct method for measuring hemoglobin attached to the insoluble membranes is described. The amount of hemoglobin found with the membrane is so regular and so large ($\pm$ 50 per cent of dry weight) as to indicate that the two are somehow bound.

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STUDIES ON THE COLLAGEN AND ELASTIN CONTENT OF THE HUMAN LUNG * †

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Connective tissues are important to the structure of all organs. Because ventilation imposes mechanical stress, the lungs are perhaps more dependent on connective tissue proteins for the maintenance of normal structure than are other vital organs. Surprisingly, the quantities of fibrous proteins in the lungs had not been measured until recently (1, 2). The purpose of this study was to determine the collagen and elastin content of human lungs. Variations in the age and sex of the subjects have been correlated with the findings.

MATERIAL AND METHODS

The right middle lobe was studied because it is an easily identified and complete unit of lung, and its size is convenient. The specimens were obtained at autopsy from subjects who did not die of diseases primarily involving the lungs. The right middle lobe bronchus was transected just proximal to its initial bifurcation leaving minimal hilar material attached to the specimen. Lobes were rejected if obvious parenchymal disease was present or if there was gross thickening of the pleura. The samples were stored at −4° C.

Measurements of collagen and elastin followed the technique of Lowry, Gilligan and Katersky (3). In brief, this procedure is gravimetric and depends upon the insoluble nature of the fibrous proteins. Finely divided tissue is extracted at room temperature in 0.1 N sodium hydroxide solution, alcohol-ether and ether. The constant dry weight of residue is obtained after separation in the centrifuge. Collagen is then converted to a soluble form with heat in the presence of water. The insoluble residue is again separated and dried to constant weight. Extraction is then carried out in 0.1 N sodium hydroxide solution in a boiling water bath. The residual material (elastin) is washed and its dry weight obtained.

It has been necessary to modify this method for use with lung tissue. Instead of being finely minced, the tissue was homogenized in water in a "Virtis 45" homogenizer. This permitted the accurate determination of dry solids on aliquots from the homogenate. One part of N sodium hydroxide solution was then added to nine parts of the homogenate. Lung collagen was converted to gelatin in the autoclave at 30 pounds of pressure for six hours. Studies of the material solubilized in this manner revealed a 17.5 per cent nitrogen and 10.5 per cent hydroxyproline content. These data characterize the conversion of collagen to gelatin and confirm the fact that no large amount of material other than collagen entered solution at this step. Reticulin would not be detected with the methods used and thus is a possible exception.

The final heated alkali extraction was continued for 45 instead of 20 minutes and the samples subsequently were washed at least twice. The material removed at this stage averaged 1.2 per cent of the initial total dry solids.

Elastin for reference was prepared from bovine neck ligament by the methods of Richards and Gies (4) and Lowry and associates (3). Table I presents the results of nitrogen analyses on bovine elastin. The mean nitrogen content of purified bovine elastin was 16.34 per cent on a dry, ash-free basis when prepared by the method of Richards and Gies (4). Although this nitrogen percentage is lower than measurements reported by others (4–7), it was determined with a procedure identical to that employed on the lung elastin residues. For this reason, it has been used for the calculation of lung elastin throughout this report. It is not inferred that human lung and bovine neck ligament elastins necessarily have identical amounts of nitrogen.

The final lung residues contained approximately 11 per cent insoluble material which was not elastin. This undoubtedly included carbonaceous material, silica and perhaps insoluble calcium salts. In order to estimate elastin, the nitrogen content of each final residue was determined in duplicate with a semi-micro Kjeldahl method. Aliquots of each digestion product were analyzed in duplicate. The mean differences in duplicate analyses were 0.07 mg. for the same digestion product and 0.09 mg. of nitrogen per 50 mg. of residue for different digestions.

To determine that the material measured by the Lowry method was elastin, the residues were exposed to a commercial preparation of crystalline elastase.[1] Duplicate lung elastin samples (weighing 50 to 100 mg.) were incubated at 37° C. with 2 mg. of elastase in 10 ml. of carbo-

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‡ Investigator of the Arkansas Heart Association.

[1] Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE I

Nitrogen determinations on bovine neck ligament elastin

Elastin preparation	Number of digestions	Number of distillations	Mean nitrogen*	Standard error
			%	%
Richards†	14	29	16.34	±0.32
Lowry‡	12	24	16.51	±0.30

* Expressed free of ash.
† Reference 4; ash—0.01 per cent.
‡ Reference 3; ash—0.82 per cent.

nate buffer at pH 8.8, ionic strength 0.1. This was continued for 24 to 48 hours or until the solution was homogeneous. The final residue was then separated by centrifugation, washed with water and dried to constant weight. The material solubilized was considered as elastin. Attempts to estimate elastin with elastase on fractions other than the final residue were unsuccessful.

Figure 1 compares the results obtained from nitrogen determinations to the results of elastase solubilization. In 30 of the present cases, the mean elastin purity of the final residues was 89 per cent by nitrogen analysis and 90 per cent by elastase solubility. The high degree of positive correlation ($r = +0.88$) between the results of the two methods offers an assurance that similar material was being measured in each instance.

Hydrolysates of the final lung elastin residues were prepared in 6 N hydrochloric acid by heating for four hours at 135° C. in sealed tubes. Hydroxyproline was then measured by the method of Stegemann (8). This measurement was to detect contamination of the final residue by collagen. The mean hydroxyproline content of lung elastin in 31 of the present cases was 1.73 per cent. This is similar to the values reported for purified elastin. Had these preparations contained any significant amount of collagen, a higher concentration of hydroxyproline would have occurred.

Hydrolysates of the final residues were also subjected to two-dimensional paper chromatography. The solvents were phenol-water (80:20) and *sec.*-butanol-formic acid-water (75:15:10). A solution of ninhydrin and isatin (9) was used to develop color. The patterns of amino acids for human lung elastins were closely similar to those obtained with bovine neck ligament elastins.

When purified bovine neck ligament elastin was analyzed with the Lowry method (3), approximately 90 per cent of the initial dry weight was recovered in the final elastin residue. Moreover, the analysis of three dog aortas with the present technique revealed a mean of 22.8 per cent of the initial dry solids each for collagen and elastin. These results agree well with the values of 25.5 per cent collagen and 25.2 per cent elastin for corresponding parts of the aorta as calculated from the data of Harkness, Harkness and McDonald (10).

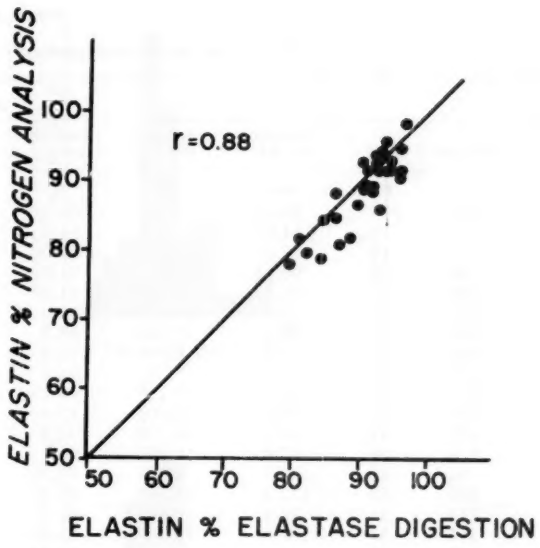

FIG. 1. PURITY OF FINAL LUNG ELASTIN RESIDUES

Elastin is expressed as per cent of dry residue weight. It was determined by the methods described in the text.

RESULTS

Table II presents data on the right middle lobes from 48 subjects. Figure 2 presents the frequency distribution for age, total middle lobe collagen, total elastin and the ratio of collagen to elastin. Figure 3 (A and B) shows collagen plotted against age. These graphs fail to reveal any significant change in lung collagen with advancing age. There was no significant difference in total middle lobe collagen between men and women. A discrepancy existed in the percentage of collagen which was related to the smaller weight of the middle lobe in women. The mean wet and dry weights, respectively, of the right middle lobe were 48 and 8.2 Gm. for women, and 76 and 13.5 Gm. for men.

Elastin, shown in Figure 4 (A and B), increased with age whether considered as a percentage of initial dry weight or as total amount. The women had a higher percentage of elastin than the men, but the total quantity of middle lobe elastin did not differ between the sexes.

The various correlations with age are shown in Table III. The three small children in the series were excluded from the calculation of the correlation coefficients in order to avoid the undue influence of these extreme values. The ratio of lung

TABLE II

Collagen and elastin measurements on the right middle lobes of 48 subjects

Autopsy	Age	Sample* wet wt.	Sample* dry wt.	Residue purity†	Elastin	Collagen	Ratio C/E§	Diagnosis
				Males				
No.	*Yrs.*	*Gm.*	*Gm.*	*%*	*%‡*	*%‡*		
169	3	26.05	4.702	81.3	4.19	10.71	2.56	Aortic repair (post-op.)
266	19	54.93	11.414	64.6	2.31	13.68	5.94	Stab wound (neck)
2	19	67.6	13.189	87.0	2.06	8.64	4.19	Cerebral contusion
16	28	90.11	11.669	91.9	3.57	14.34	4.01	Sickle cell anemia
168	31	124.1	26.198	81.6	3.07	7.32	2.38	Crushing injury
423	38	70.5	14.579	94.0	3.07	10.58	3.45	Myocardial infarction
240	39	88.4	17.044	88.3	4.75	14.74	3.10	Coronary atherosclerosis
360	43	42.9	8.541	78.8	3.54	19.08	5.37	Hemorrhage (peptic ulcer)
197	44	31.6	7.325	91.1	5.86	14.12	2.42	Subarachnoid hemorrhage
111	45	116.7	20.166	98.1	3.19	8.99	2.82	Status epilepticus
249	45	89.0	15.602	85.9	3.35	8.67	2.59	Myocardial infarction
432	47	81.5	18.378	92.0	3.89	10.02	2.57	Gun shot wound (head)
234	48	67.0	11.899	96.7	4.75	12.49	2.63	Status epilepticus
94	48	38.5	8.004	87.9	6.78	18.42	2.72	Glomerulonephritis
104	50	131.2	24.574	89.6	2.80	9.97	3.55	Myocardial infarction
353	53	76.4	13.362	91.7	4.15	9.25	2.23	Dissecting aneurysm
341	57	107.8	18.542	84.5	2.57	5.93	2.30	Leukemia
178	62	80.4	15.051	92.4	5.69	9.05	1.59	Bacterial endocarditis
65	64	45.1	8.307	89.2	6.46	13.81	2.14	Diabetes mellitus
162	64	96.3	13.492	93.3	4.71	9.36	1.99	Lymphoepithelioma
314	67	44.3	9.108	86.7	6.48	8.35	1.29	Acute peritonitis
140	69	42.7	8.655	90.5	5.85	7.85	1.34	Hemorrhage (peptic ulcer)
137	70	50.6	8.936	92.8	8.28	16.23	1.96	Myocardial infarction
276	71	118.8	16.775	81.6	3.06	6.45	2.11	Hepatoma
101	73	68.7	13.129	79.7	6.20	9.10	1.47	Coronary atherosclerosis
253	73	61.85	8.634	94.3	6.42	13.47	2.10	Hemorrhage (post-op.)
147	74	52.9	8.829	78.0	7.37	14.58	1.98	Glomerulonephritis
68	77	127.0	18.910	92.5	4.50	13.30	2.95	Myocardial infarction
268	80	77.8	10.557	91.3	6.33	13.40	2.12	Acute colitis
				Females				
284	2	17.64	3.366	62.7	1.33	7.49	5.63	Megacolon acquired
11	6	19.27	2.058	90.3	4.09	16.41	4.01	Cerebral atrophy
466	20	42.1	7.014	90.6	3.46	13.83	4.00	Ovarian carcinoma
448	30	75.7	9.001	89.5	2.96	10.70	3.83	Puerperal sepsis
403	32	34.8	5.624	88.5	6.41	19.14	2.99	Renal cortical necrosis
191	33	43.08	8.418	87.7	3.54	12.35	3.48	Subarachnoid hemorrhage
199	35	58.63	10.084	52.9	3.82	11.06	2.90	Subarachnoid hemorrhage
386	42	52.6	10.878	95.3	7.01	12.32	1.76	Nephrosclerosis
402	54	31.9	6.453	90.8	7.94	19.73	2.48	Diabetic acidosis
114	56	39.36	7.262	92.6	7.56	13.82	1.83	Esophageal stricture
481	56	50.7	7.098	90.3	8.38	20.95	2.50	Diabetic acidosis
226	57	22.73	3.966	80.8	7.64	31.16	4.08	Pyelonephritis
308	58	89.5	18.249	89.4	6.67	12.15	1.82	Cerebral hemorrhage
13	65	32.7	6.945	85.3	8.18	16.37	2.00	Verrucous endocarditis
239	67	38.37	6.960	84.3	10.70	27.50	2.57	Nephrosclerosis
446	75	84.7	12.010	86.9	8.13	16.16	1.99	Ruptured ovarian cyst
356	77	34.1	6.619	91.3	11.21	10.70	0.95	Myocardial fibrosis
480	77	36.4	5.555	86.2	8.82	15.27	1.73	Carcinoma of pancreas
463	80	49.1	7.444	90.7	10.50	14.83	1.41	Ulcerative colitis

* Right middle lobe weight.
† Calculated from analysis for nitrogen.
‡ Per cent of total intial dry solids.
§ Collagen to elastin.

collagen to elastin is plotted against age for all of the subjects in Figure 5. This ratio is useful since it excludes such variables as the weight of the lungs at the time of death and the sample size.

It was interesting that when the wet weight of the right middle lobe was 60 Gm. or less, a linear correlation was found with the dry weight which averaged 16.7 per cent of wet. Samples that weighed more than 60 Gm. wet had a smaller dry weight percentage. No relationship was evident

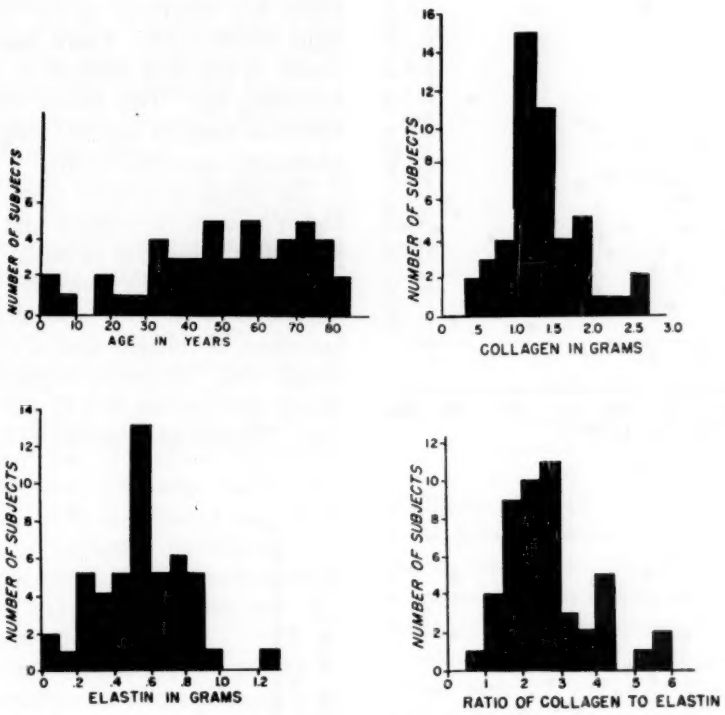

FIG. 2. FREQUENCY DISTRIBUTION OF AGE, TOTAL MIDDLE LOBE COLLAGEN, TOTAL MIDDLE LOBE ELASTIN AND THE RATIO OF COLLAGEN TO ELASTIN IN THE MIDDLE LOBE

between the age of the subject and the weight of the right middle lobe. In the whole series, the right middle lobe averaged 6.5 per cent of the total weight of both lungs as measured by the pathologist. This percentage did not vary with age.

DISCUSSION

A question of basic importance concerns the precise nature of the substances measured with the present method. The initial alkaline extractions make it unlikely that protein complexes other than collagen, elastin and reticulin deserve serious consideration. Generally accepted terminology includes as collagen the material insoluble in dilute sodium hydroxide at room temperature but converted to a soluble form with heat in the presence of water. Elastin is the residual insoluble protein after these procedures and an additional heated alkali extraction.

It must be emphasized that both collagen and elastin are complex proteins. Collagen fibers contain one or more mucoprotein fractions while elastin fibers contain mucoprotein and probably lipid fractions. A small amount of collagen also exists in a soluble form. This has been termed procollagen, and would not be measured by the present method. The fate of reticulin in this method is uncertain. Although it resembles collagen more closely than elastin, at least a part of reticulin may remain insoluble in boiling water (11). Care was taken to insure the purity of the

TABLE III

Correlation coefficients (r) with age for elastin and the ratio of collagen to elastin in adult subjects

	No.	Elastin	Elastin	Ratio
		%*	Gm.†	C/E‡
All cases	45	+0.66	+0.55	−0.73
Men	28	+0.67	+0.50	−0.64
Women	17	+0.89	+0.62	−0.84

* Calculated from dry weight of initial solids.
† Total right middle lobe elastin in dry grams.
‡ Collagen to elastin.

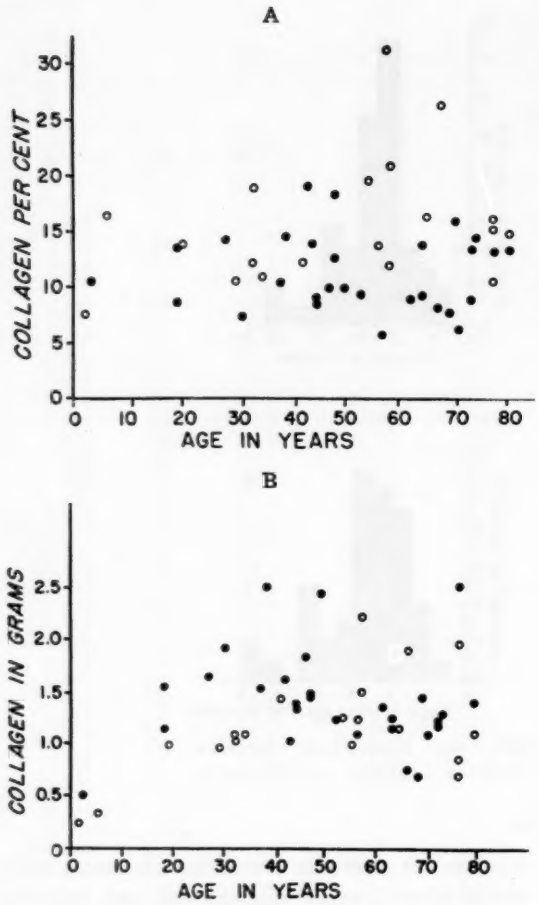

FIG. 3. SCATTERGRAMS OF COLLAGEN WITH AGE

A. Collagen expressed as per cent of the initial dry weight of the right midle. lobe. **B.** Total middle lobe collagen in grams dry weight. The open circles refer to women; the closed circles refer to men.

material reported here as elastin. Thus the lung elastin residues were found to enter solution on exposure to elastase. They contained less than 2 per cent of hydroxyproline, and had an amino acid composition similar to that found in purified bovine elastin. It may be safely concluded therefore that human lungs contain no less elastin than the amount measured by the method used in this study.

Although a wide range of values was encountered, there was no significant change in lung collagen with advancing age. Elastin, however, showed a highly significant and striking increase with age whether expressed as per cent of initial dry weight or as the total elastin of the right middle lobe. There was a consistent decrease of the lung collagen to elastin ratio with advancing age. This ratio is independent of such factors at death as vascular congestion, pulmonary edema and bronchial mucus.

The lungs of women were found to have a higher content of collagen and elastin when expressed as a per cent of initial dry weight of the sample. There was, however, no significant difference between men and women in the total quantities of collagen and of elastin in the right middle lobe. The mean weight of middle lobes obtained from women was 63 per cent of that from men. Physiological studies (12, 13) indicate that

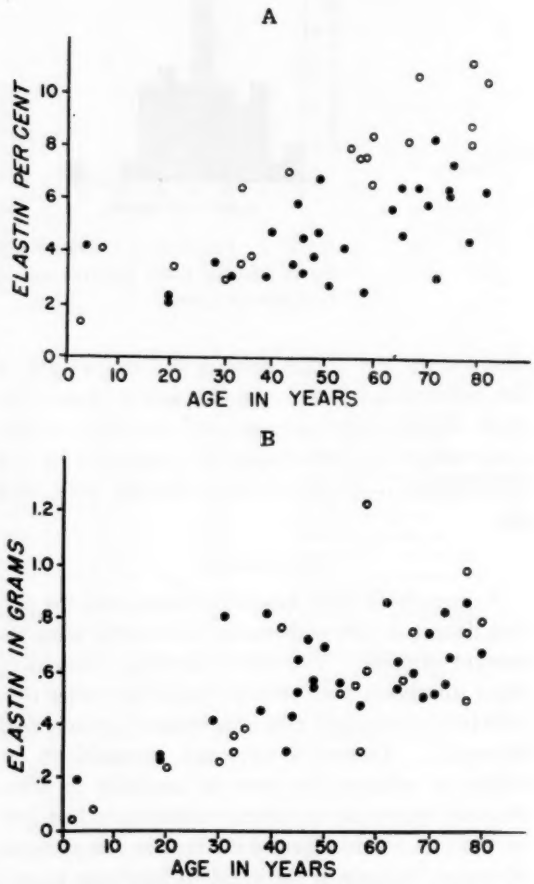

FIG. 4. SCATTERGRAMS OF ELASTIN WITH AGE

A. Elastin expressed as per cent of the initial dry weight of the right middle lobe. **B.** Total middle lobe elastin in grams dry weight. The open circles refer to women; the closed circles refer to men.

women have approximately 70 per cent of the total lung capacity found in men. Thus, it appears that women have more lung scleroprotein per unit of lung volume. Whether this observation bears any relation to the known preponderance of pulmonary emphysema in men is uncertain.

A preliminary report (1) presented analyses of the middle lobes on 20 subjects not included in this paper. The procedure was less elaborate but the results of that study agree with the present findings. Briscoe and Loring (2) measured lung elastin in 29 subjects of various ages with a modification of the Lowry method which was different from the one used in this study. These authors found an increase with age in elastin expressed as per cent of the dry weight of the sample. The values reported are somewhat higher than those reported here. The discrepancy may be related to differences in methods. Briscoe and Loring (2) found a difference in the percentage elastin between males and females which was confirmed in the present study.

Lansing, Rosenthal and Alex (14) studied the elastin of the defatted media of the pulmonary artery in 106 subjects. Those authors reported a slight increase with advancing age in elastin expressed as per cent of the defatted media. Because of the marked differences in technique, these results are difficult to compare with the present findings. Hence the influence of the vasculature on the observed changes in total middle lobe elastin cannot be assessed at the present time.

The present interest in lung collagen and elastin stemmed from a demonstration that the physical properties of the lungs were markedly different in old and young men (15). The question arose whether these changes might have resulted from alterations in the connective tissues of the lungs, especially a decrease in lung elastin. The results of the present study suggest that whatever morphologic alterations occur in the lungs of the aged, the quantity of lung scleroproteins is not decreased.

Why should elastin in the lungs increase with age? The answer is not known, but one may speculate. Bunting (16) proposed that the development of elastic fibers might be stimulated by rhythmic stress of tissue. He observed few elastic fibers in immobile scar tissues but found numerous elastic fibers in scars involving the myocardium, pericardium or pleura. He noted that,

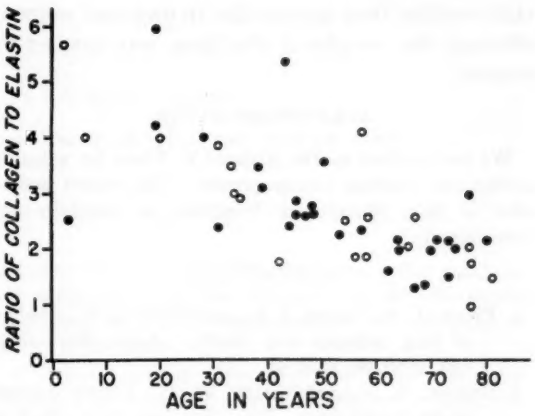

FIG. 5. SCATTERGRAM OF THE RATIO OF MIDDLE LOBE COLLAGEN TO ELASTIN WITH AGE

in adhesions, the elastic fibers were found parallel to the lines of tension. Dyson and Decker (17) also commented on the characteristic association in tissues of elastosis with fluctuating or rhythmic stress. One difficulty encountered in the interpretation of such data is the uncertainty whether stained elastic tissue is identical with the protein elastin as characterized chemically. Further, it has been proposed that collagen may be transformed into elastin. Although this would involve drastic alterations in the amino acid composition of protein molecules in the fibers, some tendency toward such a transformation has been observed *in vitro* (18, 19). Unfortunately, however, the morphologic observations (20) are not entirely specific and the chemical observations (21) are not sufficiently complete to be conclusive. Finally, it seems remotely possible that an unknown protein may exist which has an amino acid composition closely similar to but not identical with elastin. The accumulation of such a protein could account for the present findings. The available information does not permit the correct possibility to be selected at the present time.

SUMMARY

1. Collagen and elastin were measured on the right middle lobes of 48 people with a modification of the Lowry, Gilligan and Katersky alkaline extraction method (3).

2. An increase in elastin occurred with advancing age.

3. The total collagen and elastin content of the

right middle lobes was similar in men and women, although the weight of the lobes was smaller in women.

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PYRIMIDINE METABOLISM IN MAN. III. STUDIES ON LEUKOCYTES AND ERYTHROCYTES IN PERNICIOUS ANEMIA * †

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The mechanism of action of vitamin B_{12} has not been established at a biochemical level. Evidence from both animal and microbiological studies has suggested that B_{12} may serve as a cofactor in the neogenesis of methyl groups or in the formation of deoxyribose (1, 2). In such a capacity, B_{12} might be concerned with the synthesis of the methyl group of thymine (3) or of the pentose moiety of thymidylic and deoxycytidylic acids. *In vitro* studies of pernicous anemia marrow have indicated a direct stimulation by B_{12} of the incorporation of C^{14}-formate into thymine (4). The partial remission in pernicious anemia produced by certain administered pyrimidines, notably orotic acid (5), supports the hypothesis that B_{12} deficiency leads to a derangement of pyrimidine metabolism in man. Direct analyses of marrow components in pernicious anemia have demonstrated abnormal relationships of nucleic acids such that the ratio of ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) is increased (6). This report concerns studies of the cellular levels of three enzymes in pyrimidine biosynthesis using circulating erythrocytes and leukocytes from patients with pernicious anemia.

EXPERIMENTAL PROCEDURE

1. Enzymatic studies. Assays were carried out on aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase (Figure 1). The methods employed in separation of cells, sonication of leukocytes, hemolysis of erythrocytes and assay of the enzymes have been described in detail previously (7). In brief, each assay depended on the incorporation of a C_{14} precursor of high specific activity into a product which was then isolated and determined by the carrier technique. For comparative purposes results are expressed as millimicromoles (mμMoles) of product synthesized per 10^8 leukocytes or per 10^9 erythrocytes.

2. Patients studied. Studies were carried out on 10 patients with pernicious anemia in relapse. (Incomplete studies were carried out on an additional patient.) The diagnoses were based on the cytological findings in blood and marrow and specific response to B_{12} therapy. In some instances, the diagnosis was further supported by serum B_{12} levels and positive Schilling tests. Hemoglobin levels averaged 6.3 Gm. per cent (range 3.7 to 8.5).

RESULTS

Aspartate carbamyltransferase

Aspartate carbamyltransferase, the first enzyme unique to pyrimidine biosynthesis, catalyzes the irreversible carbamylation of L-aspartic acid by carbamylphosphate to form carbamylaspartic acid (Figure 1) (8). A summary of the enzymatic activity of aspartate carbamyltransferase in normal erythrocytes and leukocytes and in the abnormal blood cells of 10 patients with pernicious anemia is presented in Figure 2A. Included for comparative purposes are studies of erythrocytes of one patient with the Di Guglielmo syndrome and the leukocytes of five patients with myelocytic leukemia. More detailed information concerning pyrimidine metabolism in leukemic cells is presented elsewhere (9). A considerable variation was found in cellular enzyme levels in pernicious anemia, but in both erythrocytes and leukocytes the activities were significantly greater than in normal cells ($p < 0.01$). Pernicious anemia erythrocytes averaged higher activities than those found in the immature erythrocytes of a patient with the Di Guglielmo syndrome with reticulocytosis (9 per cent) and circulating nucleated erythrocytes (55 per cent of nucleated cells). In some patients with pernicious anemia, the leukocyte aspartate carbamyltransferase was as elevated as that found in myelocytic leukemia.

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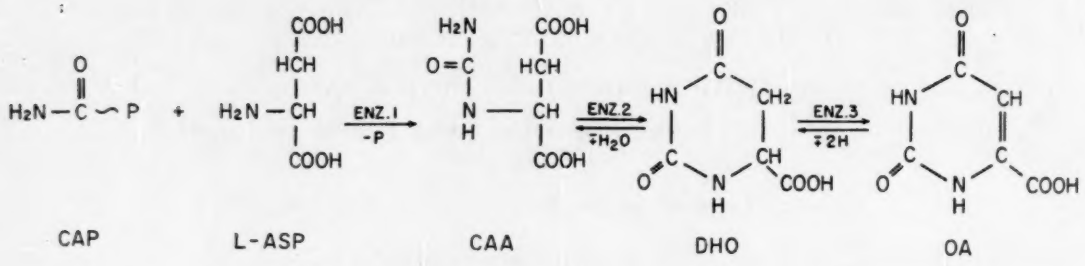
† A preliminary report of this work has been published in abstract form (Pyrimidine studies in pernicious anemia. J. clin. Invest. 1959, **38**, 1044).



ENZ. 1 - ASPARTATE CARBAMYLTRANSFERASE
ENZ. 2 - DIHYDROOROTASE
ENZ. 3 - DIHYDROOROTIC DEHYDROGENASE

FIG. 1. PYRIMIDINE SYNTHETIC ENZYMES STUDIED IN BLOOD CELLS
CAP, carbamylphosphate; L-ASP, L-aspartic acid; CAA, carbamylaspartic acid; DHO, dihydroorotic acid; OA, orotic acid.

Dihydroorotase

Dihydroorotase catalyzes the reversible cyclization of carbamylaspartic acid to form dihydroorotic acid (Figure 1) (10). The biochemical characteristics of this enzyme in human blood cells have been described elsewhere (7). In Figure 2B a summary is given of the enzymatic activities of dihydroorotase found in the erythrocytes and leukocytes of pernicious anemia in comparison with normal enzyme levels and in contrast to the values in the Di Guglielmo syndrome and myelocytic leukemia. Despite the variations in individual patients, the enzyme levels are again significantly increased ($p < 0.01$) in both erythrocytes and leukocytes. In several patients with pernicious anemia, leukocyte dihydroorotase was found to be within the range of myelocytic leukemia.

Dihydroorotic dehydrogenase

Dihydroorotic dehydrogenase catalyzes the reversible oxidation of dihydroorotic acid (DHO) to orotic acid (OA) as shown in Figure 1 (11). In previous studies this enzyme was found to be absent in mature erythrocytes and present in reproducible activity in leukocytes (7). By cell fractionation studies the enzymatic activity was found to be largely in the cell nucleus. In Figure 2C the levels of dihydroorotic dehydrogenase activity are summarized for pernicious anemia, normal cells, Di Guglielmo syndrome and myelocytic leukemia. In the erythrocytes of pernicious anemia, dihydroorotic dehydrogenase could not be detected, as it can be, in normal erythrocytes. In contrast, the abnormal nucleated erythrocytes of the Di Guglielmo syndrome exhibited this activity. In the leukocytes of pernicious anemia, dihydroorotic dehydrogenase activity was normal, except for one deviant value. This is in contrast to the marked increase of activity noted in leukemic cells.

Reversibility of the enzyme abnormalities

All of the patients studied responded hematologically to parenteral B_{12} therapy. In two of the patients, follow-up studies were carried out after hematological remission was complete. Enzymatic activities of aspartate carbamyltransferase and dihydroorotase returned to normal (Figure 3). The time course of this "enzymatic remission" was not followed because of the complications introduced by reticulocytosis and variations in cell age.

Relationship of enzyme abnormalities to the severity of pernicious anemia

An attempt was made to demonstrate some relationship between the increase of enzymatic activities and the severity of pernicious anemia, as indicated by the level of the initial hemoglobin. Although, in general, patients with the severest anemia seemed to have the greatest cellular enzymatic alterations, this relationship was not well defined. This is perhaps not surprising in view of the variation in the systemic response of patients to B_{12} deficiency—variations in the relative de-

gree of neurological, epithelial and hematological abnormalities.

DISCUSSION

Increased activities of aspartate carbamyltransferase and dihydroorotase were consistently found in both the erythrocytes and leukocytes of patients with pernicious anemia in relapse. There is no evidence from extensive animal and bacteriological studies that vitamin B_{12} is directly concerned in the synthesis or action of these two enzymes (1, 12). The first possibility to be considered is that

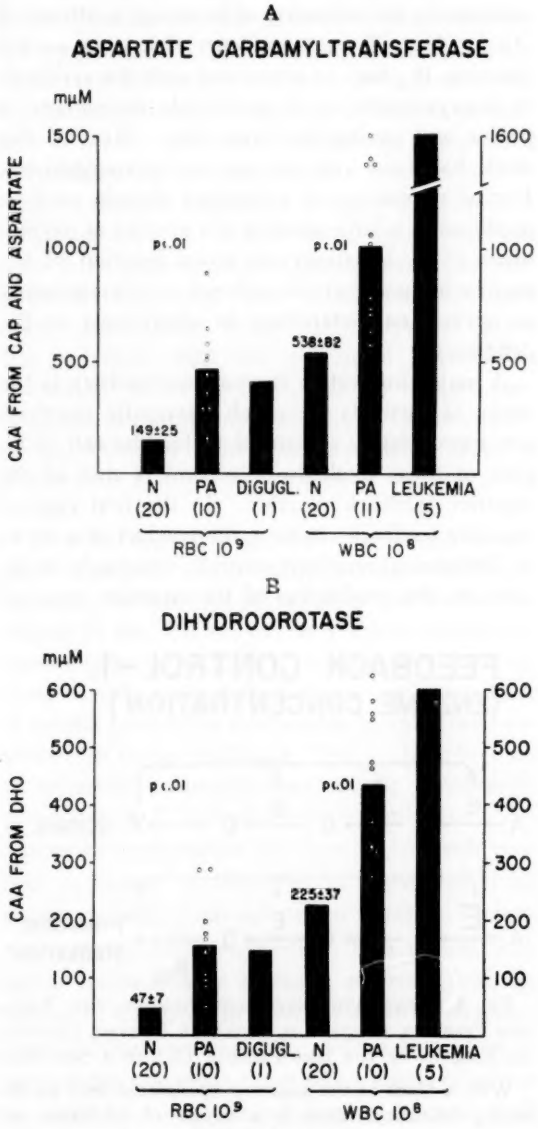


FIG. 2—*Continued*

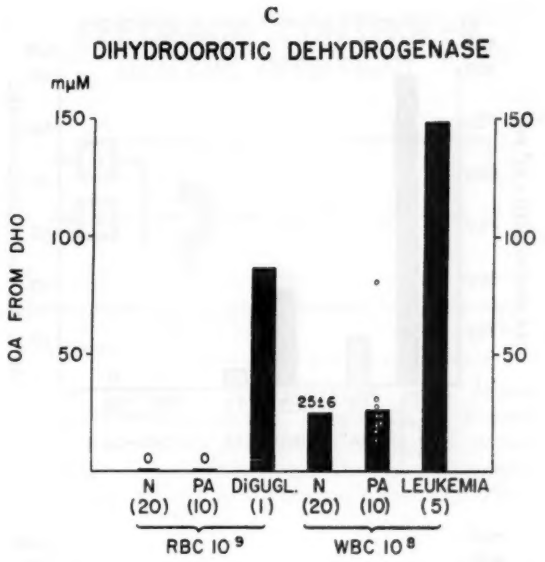


FIG. 2. LEVELS OF ASPARTATE CARBAMYLTRANSFERASE (A) DIHYDROOROTASE (B), AND DIHYDROOROTIC DEHYDROGENASE (C), RESPECTIVELY, IN THE LEUKOCYTES AND ERYTHROCYTES OF PERNICIOUS ANEMIA

These levels are compared with those found in normal cells, leukemia and the Di Guglielmo syndrome.

the enzymatic abnormalities found represent a nonspecific increase of enzyme levels in young cells. It has been clearly shown that pernicious anemia is, in part, a hemolytic anemia with increased destruction of erythrocytes in the peripheral blood and probably within the bone marrow (13). The circulating macrocyte is therefore a young cell. Evidence from several sources suggests that immaturity of cells does not account for the increase of enzyme activity observed. There is no cytological evidence of immaturity of circulating polymorphonuclear leukocytes in pernicious anemia. The enzymatic activities of aspartate carbamyltransferase and dihydroorotase observed were comparable to those found in the Di Guglielmo syndrome and myelocytic leukemia, conditions of much greater immaturity of cells as judged by cytological criteria. Finally, the pattern of alteration of enzyme activity in the leukocyte was not that found in immaturity *per se*. An increase of dihydroorotic dehydrogenase activity is the most striking abnormality of the immature leukocyte, as demonstrated in myelocytic leukemia and in the leukocytosis of infection (9). In pernicious anemia leukocyte dihydroorotic dehydrogenase ac-

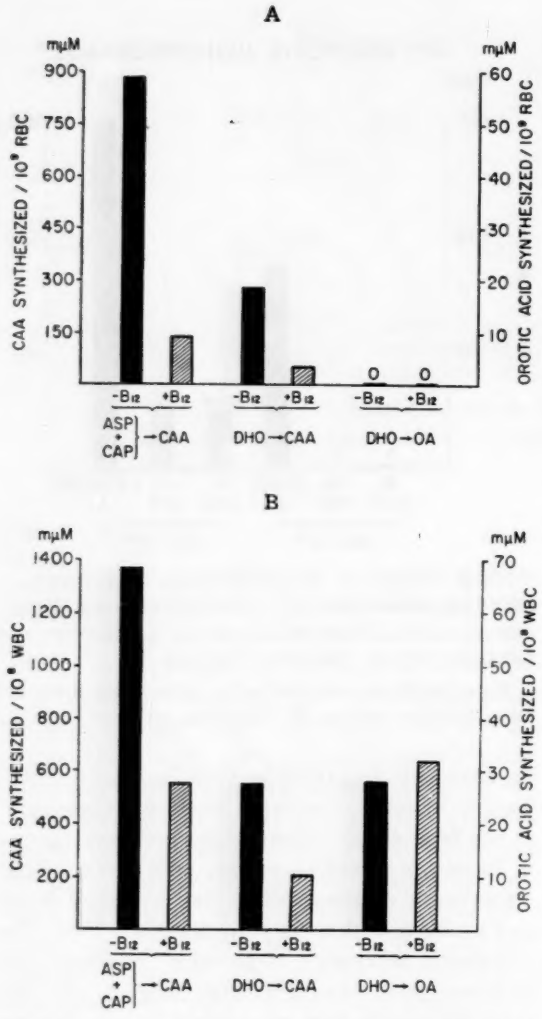


FIG. 3. ENZYME LEVELS IN THE ERYTHROCYTES (A), AND LEUKOCYTES (B), RESPECTIVELY, OF A PATIENT WITH PERNICIOUS ANEMIA BEFORE AND AFTER THERAPY WITH VITAMIN B_{12}

Before treatment Hgb. was 4.6 Gm. per cent; three months after treatment, Hgb. was 12.4. ASP + CAP → CAA, aspartate carbamyltransferase; DHO → CAA, dihydroorotase; DHO → OA, dihydroorotic dehydrogenase.

tivity is normal. In brief, there is a consistent cellular abnormality of certain pyrimidine synthetic enzymes in pernicious anemia in a pattern different from that of immaturity of cells *per se*. The altered enzyme pattern returns to normal following treatment with vitamin B_{12}.

It would be beyond the scope of this discussion to review current theories of the mechanism of action of vitamin B_{12}. The function of B_{12} appears to vary in different organisms. One of its most firmly established metabolic roles relates to the neogenesis of methyl groups in certain microorganisms (3) and animals (1). In this way B_{12} might be concerned with the synthesis of thymine, which has been reported to produce a partial remission in pernicious anemia when given in large oral doses (14). In marrow cells from pernicious anemia studied *in vitro*, the addition of B_{12} to the plasma suspension has been shown to stimulate the incorporation of C^{14}-formate into thymine, presumably an indication of increased synthesis of DNA (4). There is evidence that as a second function, B_{12} may be concerned with the synthesis of deoxyriboside or deoxyribotide derivatives of purine and pyrimidine bases (2). Most of this work has been carried out on microorganisms. Partial remissions of pernicious anemia may be produced by a large amount of a number of pyrimidines (5). Whatever the exact function of B_{12} may be in man, there is evidence of a derangement in pyrimidine metabolism in blood cells in B_{12} deficiency.

A major interest in current biochemistry is the study of methods by which enzymatic reactions are appropriately controlled within the cell (15). One of these methods of control is that of the negative feedback reaction. In the first type of negative feedback reaction, the product of a series of biochemical reactions controls, seemingly by inhibition, the production of the enzymes involved

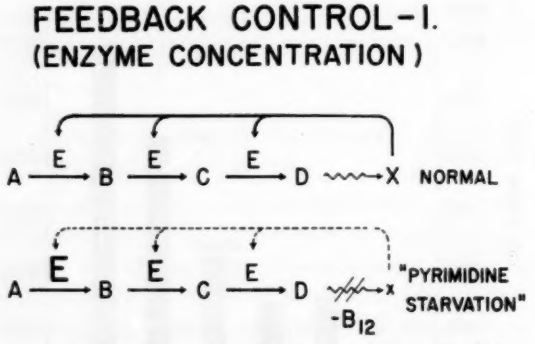


FIG. 4. SCHEMATIC REPRESENTATION OF THE NEGATIVE FEEDBACK CONTROL OF PROXIMAL ENZYME SYNTHESIS EXERTED BY THE END PRODUCT (X) IN A SEQUENCE

With a block in the sequence (postulated here as due to B_{12} deficiency) there is a release of inhibition and compensatory "hypertrophy" of the proximal enzymes.

in its own biosynthesis (Figure 4). This controlling mechanism has been clearly shown to be operative in pyrimidine synthesis in bacteria by Yates and Pardee (16). When there is a genetic block in pyrimidine synthesis, bacterial growth continues only if a pyrimidine precursor distal to the missing enzyme is furnished in the medium. If this precursor is withdrawn, there is "pyrimidine starvation" with an accompanying increase of the enzymatic activities proximal to the blocked step. It has been suggested that a fall in the concentration of the end product releases an inhibition which governs the rate at which earlier enzymes are synthesized. The resulting increase in enzyme concentration would serve as a homeostatic mechanism to maintain the optimal rate of production of the end product. The pattern of increased enzyme activity in bacteria with a block in pyrimidine synthesis is: aspartate carbamyltransferase > dihydroorotase > dihydroorotic dehydrogenase. This is the pattern found in pernicious anemia, and is the obverse of that found in immaturity of blood cells. The current studies are interpreted as being consistent with the pattern of "pyrimidine starvation" in human blood cells, in which a partial block in pyrimidine synthesis occurs secondary to B_{12} deficiency rather than a complete block in *Escherichia coli* due to the genetic ablation of an enzyme.

A second type of negative feedback reaction has been described in pyrimidine synthesis in *E. coli* in which the product serves as a competitive inhibitor of the first enzyme in the biosynthetic sequence (Figure 5) (17). This is inhibition of enzyme activity rather than enzyme concentration. A similar controlling mechanism has also been described in purine synthesis (18). The inhibition of aspartate carbamyltransferase by cytidine-5'-phosphate in *E. coli* was readily confirmed. Attempts to demonstrate this type of feedback control in human erythrocytes or leukocytes with either cytidine-5'-phosphate or uridine-5'-phosphate have been unsuccessful. In a patient with orotic aciduria large amounts of orotic acid appeared in the urine in association with refractory megaloblastic anemia (19). When a mixture of nucleotides was given there was a sharp reduction in orotic aciduria. This reduction in orotic acid synthesis was probably mediated by one or both of the negative feedback control mechanisms outlined above.

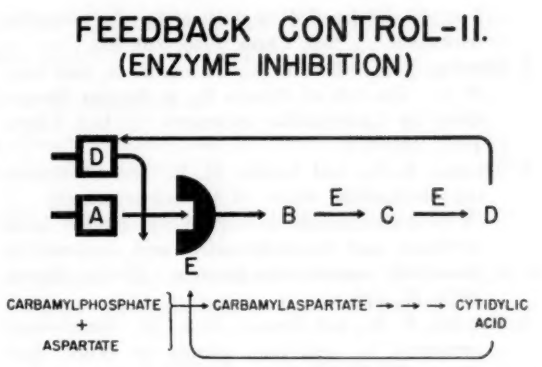


FIG. 5. SCHEMATIC REPRESENTATION OF A SECOND NEGATIVE FEEDBACK CONTROL MECHANISM IN PYRIMIDINE SYNTHESIS, THE COMPETITIVE INHIBITION OF THE FIRST ENZYME IN THE SEQUENCE, ASPARTATE CARBAMYLTRANSFERASE, BY A LATER PRODUCT, CYTIDINE-5'-PHOSPHATE

SUMMARY

1. Three enzymes leading to the synthesis of orotic acid—aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase—were studied in the erythrocytes and leukocytes of 10 patients with pernicious anemia in relapse.

2. Increased activities of aspartate carbamyltransferase and dihydroorotase with normal activity of dihydroorotic dehydrogenase were found. This pattern differs from that found in immaturity of cells *per se*. The abnormal pattern returned to normal following treatment with vitamin B_{12}.

3. It is suggested that the enzyme abnormalities are most consistent with the pattern of "pyrimidine starvation" produced by vitamin B_{12} deficiency, the indirect evidence of a negative feedback control of enzyme concentrations in blood cells.

4. A second negative feedback control mechanism, the competitive inhibition of aspartate carbamyltransferase by cytidine-5'-phosphate, was confirmed in *Escherichia coli* but could not be demonstrated in erythrocytes or leukocytes.

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ANNOUNCEMENT

A Grant Award for travel funds to the First International Congress of Nephrology in Geneva, Switzerland, September 1, 2, 3, 1960, has been awarded by the U.S.P.H.S. to the American Society for Clinical Investigation.

Members and nonmembers are eligible to apply for travel funds to the Congress. Funds are available to send 20 individuals to the meetings.

Applications for funds should include *in quadruplicate:*

a) Letter of application stating interest in the field and in the Congress.

b) Curriculum vitae.

The deadline for receipt of *a*) and *b*) is March 1, 1960. Awards will be made around March 15, 1960.

Applications should be sent to Saul J. Farber, Secretary, American Society for Clinical Investigation, New York University College of Medicine, 550 First Avenue, New York 16, New York.

REVERSIBILITY OF RENAL ATROPHY CAUSED BY UNILATERAL REDUCTION OF RENAL BLOOD SUPPLY *

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(Submitted for publication June 30, 1959; accepted September 4, 1959)

Experimental unilateral reduction of renal blood flow results in atrophy of one kidney and hypertrophy of the other (1–4). The atrophic process consists of involution of the glomeruli and transformation of the tubules into thick compact cords along with failure of excretory function. Hypertension is generally, but not necessarily, associated with unilateral partial atrophy (5); when it is present and severe, only the nonatrophic kidney shows nephrosclerosis (1–4). Remission of hypertension following restoration of renal blood flow or uninephrectomy depends on the extent of the secondary hypertensive changes in the contralateral kidney (6).

These manifestations constitute the experimental counterpart of those seen in clinical unilateral renal artery disease. Removal of the atrophic kidney is the treatment commonly advocated (7, 8). It occurred to us that if we could demonstate that a kidney atrophic, but otherwise devoid of lesions, could regain its normal excretory function, then it might be desirable to re-establish surgically its normal blood supply and if necessary to remove the contralateral kidney.

Two previous observations suggest that recovery of function may occur. In rats, unilateral curtailment of renal blood flow associated with simultaneous contralateral nephrectomy prevented renal atrophy in some of the animals (9). In dogs, a kidney anuric as a result of a clamp on the renal artery resumed urine formation after contralateral nephrectomy (10).

The purpose of the present investigations was to extend these studies to see whether renal atrophy can be prevented regularly by simultaneous contralateral nephrectomy and whether kidneys with various degrees of atrophy can recover equally well when the clamp is removed or left in place. We also observed the effects of these various manipulations on blood pressure.

* This investigation was supported in part by a grant (H-96) from the National Heart Institute, National Institutes of Health, Bethesda, Md.

MATERIAL AND METHODS

The procedure best suited for our purpose would have been one which regularly elicited a predictable degree of renal atrophy without necrosis and permitted easy removal of the constriction. Four methods were assessed: a loose nylon ligature around the aorta in very young rats (5); aortic constriction by silk thread in adult rats (1); use of silver clips on the aorta or on the renal artery (2, 6). We found that thread ligature gave a more reproducible degree of constriction but had the disadvantage of unavoidable fatal hemorrhages when release of the constriction was attempted; silver clips on aortae gave less predictable results but could be opened easily; constriction of an artery as small as the renal artery with clips was too capricious, although it permitted blood pressure measurements by tail plethysmography. For most of our experiments we adopted the procedure of aortic stenosis with silver clips.

Sprague-Dawley rats, weighing 40 to 80 Gm. were used; they were males or females since sex has no influence on the degree of renal atrophy (1). They were given tap water and fed Purina® Fox Chow. Silver clips were prepared according to the method of Wilson and Byrom (6). From silver ribbon (0.8 mm. wide and 0.13 mm. thick) which was annealed by careful heating over a gas burner, strips about 1 cm. long were cut and bent carefully over the mandrel of a No. 18 gauge needle, which gave an opening with a diameter of 0.55 mm. Following ether anesthesia and a midline incision, the aorta between the origins of the two renal arteries was dissected free with cotton pads; a clip was then hooked in this space and closed by twisting both ends together.

In one experiment "endocrine" kidneys were prepared according to the technique of Selye and Stone (1). Aortic constriction was produced in adult rats (130 to 160 Gm.) by a silk ligature which enclosed the artery and the mandrel of a No. 22 gauge needle (0.25 mm. diameter) which was subsequently withdrawn; ligature of the left ureter was done to ascertain absence of excretory function. Rats showing hydronephrosis at autopsy were discarded. Whether "endocrine" kidneys secrete excessive amounts of pressor substances (1) or, from morphologic observations (11), are deficient in antihypertensive principle is irrelevant; the fact remains that such atrophic kidneys cause an acute type of hypertension and vascu-

lar disease, associated with cachexia and high mortality.

Animals were kept after operation for periods up to 54 days. At the end of the experiment, blood was taken for blood urea nitrogen (BUN) determinations. Blood pressure was measured by tail plethysmography in rats in which the aortic clip was removed (12); in the others, hypertension was ascertained at the end of the experiment either by direct measurement in the carotid artery under light ether anesthesia or on the basis of cardiac ventricular hypertrophy. The results from these three methods are sufficiently accurate to provide comparative information on normotensive or hypertensive states: tail pressure correlates closely with carotid pressure (12) and changes in cardiac ventricular weights expressed in percentage of body weight parallel those in tail pressure (13).

At autopsy, kidneys and hearts were removed, fixed in Susa fluid, dissected and weighed; hearts were weighed after removal of auricles; sections were stained with periodic acid-fuchsin (PAS). Weights of organs were expressed in percentage of body weight at the time of removal. Since both kidneys from the same animal had nearly the same weight, we used the ratio *left kidney/right kidney* as an index of differential weight between the two kidneys; this ratio will be referred to as the *kidney ratio*. Keeping in mind that renal atrophy was always elicited on the left side, a decrease in ratio will reflect atrophy of the left kidney and/or hypertrophy of the right kidney; conversely an increase in ratio will indicate hypertrophy of the left kidney and/or atrophy of the right kidney. Interpretation of the ratio will depend on comparison with suitable controls.

EXPERIMENTAL PROCEDURE AND RESULTS

1. Effects of uninephrectomy and unilateral "endocrine kidney" on the contralateral kidney

These preliminary experiments were carried out for the following purposes: first, to ascertain the variability of the normal kidney ratio in our strain of rats; secondly, to determine the degree of compensatory hypertrophy resulting from unilateral nephrectomy and lastly, using rats with "endocrine kidney" to determine whether renal hypertrophy is solely dependent on excretory function of the contralateral kidney.

Thirty rats weighing 130 to 160 Gm. were divided into three groups: animals of Group I were controls, those of Group II were uninephrectomized, and those of Group III were operated upon as described in the "endocrine kidney" technique. Groups II and III were sacrificed seven days later. The results (Table I) show that in normal rats there is a high degree of uniformity in the weight of each kidney which is reflected in the value of the kidney ratio of 0.99. Uninephrectomy of seven days' duration elicits a statistically significant compensatory hypertrophy; the weight of the right kidney increased from 0.47 to 0.56 Gm. giving a kidney ratio of 0.83. Heart weight was unchanged. The 12 rats subjected to the "endocrine kidney" procedure lost weight from a mean value of 147 to 114 Gm. At autopsy, nine rats showed typical "endocrine" left kidneys without hydronephrosis or necrosis. Macroscopic nephrosclerosis was seen in four rats, but only in the right kidney; cardiac necrosis and hemorrhages appeared in three animals; all the rats showed microscopic nephrosclerosis. There was about 33 per cent atrophy of the left kidney (0.31 to compare with 0.47 Gm. in controls) and a corresponding hypertrophy of the right kidney; this inverse relationship gave a kidney ratio of 0.49. Hypertrophy on the right side was significantly greater

TABLE I

Effects of uninephrectomy and unilateral "endocrine kidney" on contralateral renal hypertrophy

Groups	Procedure	Kidney*		Kidney ratio† left/right ± S.E.	Heart wt.† ± S.E.
		Right	Left		
					Gm./100 Gm. body wt.
I		0.47 (0.40–0.55)	0.46 (0.40–0.54)	0.99 ±0.009	0.36 ±0.007
II	Uninephrectomy left	0.56 (0.49–0.62)	0.47 (0.44–0.53)	0.83 ±0.024	0.35 ±0.009
III	"Endocrine kidney" left	0.63 (0.58–0.70)	0.31 (0.27–0.37)	0.49 ±0.029	0.45 ±0.022

* Mean and range.
† Mean and standard error of the mean.

TABLE II

Effects of releasing aortic stenosis and uninephrectomy on kidney and heart weights (20 days after clamping)

Groups and no. of animals	Operative procedure on day				Kidney ratio† left/right ± S.E.	Heart wt.† ± S.E.	BUN‡
	1*	20	27	34			
						Gm. per 100 Gm. body wt.	*mg./100 ml.*
I 41		Killed			0.76 ±0.023	0.45 ±0.012	20.1 (11–38)
II 5		Unclamping	Killed		0.80 ±0.046	0.43 ±0.02	13.9 (11–19)
III 5		Unclamping	Right nephrectomy	Killed	1.10 ±0.052	0.43 ±0.031	12.9 (11–15)
IV 7		Unclamping + right nephrectomy	Killed		0.98 ±0.056	0.43 ±0.023	22.0 (11–53)
V 12		Right nephrectomy	Killed		0.99 ±0.035	0.43 ±0.011	30.0 (24–41)
VI 12		Left nephrectomy	Killed		0.59 ±0.033	0.36 ±0.009	25.9 (19–33)
VII 10	Right nephrectomy	Killed			1.12 ±0.038	0.44 ±0.007	

* Aortic clamping was performed in all groups.
† Mean and standard error of the mean.
‡ Mean and range.

($p < 0.05$) than after uninephrectomy; this was probably due to early nephrosclerosis which is associated with accumulation of tubular casts before severe cellular atrophy or other degenerative changes take place. The marked increase in heart weight provides another evidence of high blood pressure.

2. Effects of release of aortic constriction alone or in association with unilateral nephrectomy

A. Twenty days of aortic constriction. The purpose of this experiment was to see whether unilateral renal atrophy due to aortic stenosis could be prevented by simultaneous contralateral nephrectomy, or reversed 20 days later by release of the constriction alone or in association with contralateral nephrectomy. The general plan as well as the operative procedures and results are outlined in Table II. One hundred fifty rats of both sexes were divided into seven groups and subjected to aortic stenosis (Groups I through VI) and to aortic stenosis plus right nephrectomy (Group VII). On the twenty-first postoperative day, animals of Groups I and VII were sacrificed. Further operations as scheduled (Table II) were carried out only in those animals demonstrating on the operating table atrophy and lack of necrosis of the left kidney; 13 per cent of the survivors were discarded. Whenever release of the constriction (Groups II to IV) on the twenty-first day resulted in severe hemorrhages, such animals were sacrificed and the data added to those of the control, Group I. The experiment was terminated seven days after the last surgical intervention, since this period of time from the previous experiment was sufficient to permit significant compensatory renal hypertrophy.

Mortality for Groups I through VI following aortic stenosis amounted to 20 per cent. This was not increased by simultaneous right nephrectomy: in Group VII only one out of 11 rats died. Following nephrectomy and/or release of the clamp, mortality varied between zero and 17 per cent, except in Group IV (release of the constriction plus simultaneous right nephrectomy) where it reached the value of 46 per cent. Overall mortality and discards because of renal necrosis amounted to 46 per cent of the animals.

Comparison of normal BUN values (mean 15.9, range 13 to 19.4 mg. per 100 ml.) with those obtained in various groups showed a slight but definite increase in Groups I, IV, V and VI. Opening of the clamp was sufficient to bring BUN values to normal (13.9 mg. to compare with 20.1 mg. per 100 ml. in Group I) but not when associated with simultaneous right nephrectomy (Group IV). However, when right nephrectomy was postponed by seven days, BUN values remained normal.

Aortic constriction (Group I) resulted, as in the preceding experiment with the "endocrine kidney," in a kidney ratio of less than one which was not altered by release of the constriction (Group II). When right nephrectomy was performed at the time of unclamping (Group IV) or seven days later (Group III), the atrophic kidney enlarged and kidney ratios became equal to or greater than one. There was no statistical difference between Groups III and IV. When right nephrectomy was performed without touching the clamp (Group V), the kidney ratio was almost equal to one, indicating that kidney hypertrophy can occur in spite of partial obstruction to renal blood supply. This conclusion was confirmed by the results in Group VII in which right nephrectomy was performed at the time of aortic constriction; the left kidney not only did not atrophy but actually increased its size, with a resulting kidney ratio of 1.12. The ratio of 0.56 obtained in Group VI merely indicates that removal of a kidney made atrophic by aortic clamping can elicit further compensatory hypertrophy, presumably because some excretory function remained. Incidentally, in a few rats of Group I, the left kidney was larger than the right giving individual ratios greater than one (one of them was 1.3) in comparison with a mean ratio of 0.76 for the group; in these instances the right kidney was severely nephrosclerotic.

Heart weight values (Table II) were greater than 0.43 in all the groups except in Group VI where it was normal and equal to 0.36. This is in agreement with results of blood pressure determinations. Normal values were obtained in Group VI (mean 110, range 105 to 120 mm. Hg); in the other groups values were mostly over 150 mm. Hg and as high as 200 mm. Hg.

The localization and nature of the lesions have been amply documented by studies on rats with "endocrine kidneys" (1, 2). Except for evidence of hypertensive vascular disease in all groups, histologic examinations were not informative. We could not detect any change suggesting healing in Group VI where hypertension remitted, probably because of the short period of observation. In this group, lesions of arteritis or arteriolar necrosis were present in the heart or right kidney of six rats; however, there was none in the pancreatic or mesenteric blood vessels. It is worth noting that these lesions did not prevent the fall in pressure subsequent to left nephrectomy.

Since the period of observation after unclamping or uninephrectomy may have been too short to evaluate the complete effects of these procedures, we have, in the following experiments, extended the period from 7 to 20 days. Aortic stenosis was performed in 60 rats which were divided into five groups. Groups I and II were controls and were sacrificed, respectively, on the twentieth and fortieth days. The other groups were subjected on the twentieth day to the following operative procedures: unclamping (Group III), unclamping plus right nephrectomy (Group IV), and unclamping plus left nephrectomy (Group V). The experiment was terminated on the fortieth day. The results obtained were in agreement with the previous ones. In short, renal atrophy was more pronounced after 40 than after 20 days, as indicated by the respective values of kidney ratios of 0.65 and 0.76; unclamping did not alter the ratio (0.63), while simultaneous right nephrectomy increased its value to 1.16. The only other significant observation was the high heart weight (mean 0.41) following unclamping as compared with the normal value (mean 0.34) following removal of the atrophic kidney. Histologic examination showed that after unclamping and right nephrectomy, there was complete restoration of renal morphology so that the left kidney could not be distinguished from a normal kidney which had undergone compensatory hypertrophy.

B. Forty days of aortic constriction. The purpose of this experiment was similar to that of the previous one; the difference was in extension from 20 to 40 days of the period following aortic stenosis, in order to produce chronic renal atrophy (Table III). One hundred forty-seven rats were divided into five groups and subjected to aortic stenosis alone (Groups I to IV) or in association

TABLE III

Effects of releasing aortic stenosis and uninephrectomy on kidney and heart weights (40 days after clamping)

Groups and no. of animals	Operative procedure on day				Kidney ratio† left/right ± S.E.	Heart wt.†
	1*	40	47	54		
						Gm. per 100 Gm. body wt.
I 33		Killed			0.68 ±0.026	0.37 ±0.009
II 12		Unclamping	Killed		0.76 ±0.107	0.40 ±0.016
III 9		Unclamping	Right nephrectomy	Killed	0.90 ±0.050	0.38 ±0.019
IV 7		Unclamping + right nephrectomy	Killed		0.91 ±0.013	0.40 ±0.010
V 14	Right nephrectomy	Killed			1.03 ±0.027	0.39 ±0.007

* Aortic constriction was performed in all groups on the first day.
† Mean and standard error of the mean.

with right nephrectomy (Group V). On the fortieth day Groups I and V were sacrificed; the rats of the remaining groups (less 12 per cent discarded for renal necrosis) were operated as scheduled and sacrificed seven days after the last intervention.

Mortality during the first 40 days was 29 per cent in the groups with aortic stenosis alone; in Group V in which the animals had also right nephrectomy, mortality was 22 per cent. Subsequent surgical intervention in Groups II and IV resulted, respectively, in a mortality of 14, 17 and 42 per cent; this relative proportion corresponds to that obtained in similar groups of the 20 day experiment. BUN values were so widespread as to be noninformative.

The results on kidney ratios (Table III) show complete agreement with those obtained in the 20 day experiment. In the control, Group I, it was equal to 0.68. In some of the rats, the left kidney weighed only about 140 mg. per 100 Gm. of body weight with a corresponding value of 640 mg. for the right. Opening of the clamp (Group II) had no statistically significant effect but simultaneous (Group IV) or delayed (Group III) right nephrectomy increased the ratio to 0.91 and 0.90, respectively. The highest ratio (1.03) was obtained in Group V, where aortic clamping was associated with simultaneous right nephrectomy.

Heart weights (Table III) and blood pressure were only slightly elevated and differences between groups were not statistically significant. The contrast between these results and those of the 20 day experiment, can be explained by the fact that during the period of 40 days following aortic stenosis, more rats, not included in the table, developed severe hypertensive vascular disease and died. Therefore, only mild or no lesions in the renal and extrarenal vascular bed were seen in the survivors. Another observation was that the left kidney of the rats which died following right nephrectomy (Groups III and IV) was still atrophic with marked interstitial fibrosis and casts in the few functioning nephrons. Evidently such a kidney was too involuted and diseased to take over the excretory function of the contralateral kidney.

DISCUSSION

Under uniform conditions, total renal mass, which is equally distributed between both kidneys, tends to remain constant (14) so that unilateral atrophy such as that produced by clamping of the renal artery is accompanied by contralateral compensatory hypertrophy (2). This results in a new equilibrium between the two kidneys which is not altered by removal of the clamp. When the hypertrophic kidney is removed, the contralateral

atrophic kidney increases in size and regains its function, even when the clamp is left in place. This is demonstrated by the present experiments in which uninephrectomy was performed at the same time as, or 20 to 40 days after, contralateral reduction of renal blood flow, and is in accord with previous findings in dogs (10) and rats (9). However, absence of compensatory hypertrophy has also been reported in 5 of 14 rats (9) and in young rabbits (15) in which thread was used as constricting material. These discrepancies may be explained by the observation (5) that a loose nylon ligature around the aorta in young rats results during body growth in a progressive and almost complete involution of the left kidney without evidence of hypertension. Presumably the blood supply to this kidney, being gradually decreased, is barely enough to maintain the integrity of renal tissue and is therefore insufficient to permit hypersecretion of pressor substances or compensatory hypertrophy. Since gradual and complete renal involution was not observed with silver clips, one could assume that the physical nature of the constricting material (such as elasticity, width) is of critical importance.

The nature of the stimulus responsible for compensatory hypertrophy is unknown. The hypothesis which takes into account most of the pertinent observations has been proposed by Braun-Menendez (16). In short, kidney growth is regulated by protein metabolites (renotrophins) which are normally destroyed by the kidney; following reduction in renal mass, they accumulate and stimulate renal growth until a new equilibrium is reached. The mitosis-stimulating factor found in blood following uninephrectomy in rats may be renotrophin (17). That the kidney could inactivate its own secretions has been demonstrated with renin (18). According to this hypothesis, hypertension develops whenever a kidney cannot increase its size in order to maintain renotrophins at their normal levels. From this it is assumed that an atrophic kidney, resulting from renal artery clamping cannot hypertrophy. The present experiments disprove this assumption, but suggest that an unclamped kidney is more responsive than a clamped kidney to the same renotrophic stimulus.

Although aortic stenosis resulted generally in a kidney ratio left/right smaller than one, we found a few cases in which the left kidney was larger than the right. In such animals, the left had a normal appearance while the right was contracted and nephrosclerotic. Having eliminated anatomical anomalies, we assumed that the following events took place: aortic clamping elicited atrophy of the left kidney and severe hypertension which in turn produced nephrosclerosis of the unprotected right kidney; the ensuing loss of functioning tissue resulted in contraction of this kidney and secondarily in hypertrophy of the atrophic kidney. Since the left hypertrophied kidney remained structurally normal, the factor responsible for hypertrophy is different from the one causing nephrosclerosis. These observations also demonstrate that renal artery clamping protects the kidney against high blood pressure without interfering with renotrophic effects.

Release of the aortic constriction did not usually bring back blood pressure to normal levels as evidenced by heart weight. The lack of reliability of plethysmographic values in this situation was demonstrated by the impossibility, particularly in the 40 day experiment, in registering heart beats from the tail of some animals, suggesting persistent partial obstruction due to tissue proliferation (6). Considering heart weights greater than 0.40 Gm. per 100 Gm. of body weight as indexes of hypertension, we found that between 40 and 75 per cent of the rats were still hypertensive after release of aortic constriction. On the other hand, following removal of the atrophic kidney, the percentage of animals remaining hypertensive was less than 10 per cent. The most obvious explanations for the persistence of hypertension are presence of vascular lesions in the contralateral kidney and in the extrarenal arterial bed, and persistence of mechanical obstructions in the aorta (19). Pieces of aorta removed for histologic examination were unfortunately lost. Indeed, a slight obstruction may be sufficient to maintain hypertension without marked interference with normal renal function as demonstrated in dogs by Corcoran and Page (20).

SUMMARY

1. Unilateral renal atrophy was elicited in young rats by partial constriction of the aorta between the origins of the two renal arteries with silver clips. This was associated with uninephrec-

tomy, unclamping, or a combination of the two procedures. Effects on kidney weight were expressed by the kidney ratio, *left kidney/right kidney*, which in normal rats is equal to one.

2. Aortic stenosis alone resulted 20 and 40 days later in a kidney ratio of 0.76 and 0.68, respectively.

3. Right nephrectomy performed simultaneously with aortic stenosis did not increase the mortality over aortic stenosis alone. Left renal atrophy was prevented as shown by kidney ratios greater than one.

4. Removal of the clamp, 20 to 40 days after aortic stenosis, did not alter the kidney ratio. However, right nephrectomy caused hypertrophy of the left kidney, even when the clamp was left in place. The degree of atrophy did not influence recoverability as long as there were no degenerative lesions.

5. Unilateral renal atrophy was usually but not necessarily accompanied by hypertension. Left nephrectomy was more effective in remitting hypertension than removal of the clamp.

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SUSCEPTIBILITY OF RATS TO EXPERIMENTAL PYELONEPHRITIS FOLLOWING RECOVERY FROM POTASSIUM DEPLETION * †

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Ureteral ligation (1–3), mechanical trauma (4, 5), scar formation in the kidney after recovery from severe staphylococcal infection (6), localized thermal injury to the renal medulla (7), and renal lesions induced by desoxycorticosterone acetate (DCA)-saline hypertension (8, 9) have been shown to predispose to the development of acute, hematogenous, *Escherichia coli* pyelonephritis in the experimental animal. It is established that potassium depletion produces renal lesions in experimental animals and in man (10, 11). A *temporary* deficiency of potassium has been shown to lead to permanent renal damage in the rat despite subsequent repletion (12, 13). However, the degree of renal damage was quite dissimilar in the two studies, which has raised the possibility that spontaneous pyelonephritis may have complicated the findings in the one of these studies in which observations were not made to exclude it (12). Clinical data suggest an association between potassium nephropathy and pyelonephritis but this remains only suggestive (14, 15). There is evidence both for (15) and against such an association in the experimental animal (13, 16). The present investigation was therefore designed to test, by experiment, the hypothesis that animals which have recovered from potassium depletion may have increased susceptibility to pyelonephritis. The problem of susceptibility to infection *during* potassium depletion is under current investigation.

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MATERIALS AND METHODS

White, male Sprague-Dawley strain rats, weighing 128 to 160 Gm., were used. Potassium depletion was accomplished by means of a basal diet which was deficient in potassium, sodium, phosphate and chloride. Control groups received the following daily quantity of supplementary electrolytes by gavage: 5 cc. of gavage solution containing 2 mMoles of potassium, 2 mMoles of sodium, 2.5 mMoles of chloride and 0.75 mMole of phosphate. The potassium-deficient group received 4 mMoles of sodium, 2 mMoles of bicarbonate, 0.5 mMole of chloride and 0.75 mMole of phosphate. The method of producing potassium depletion, including content of diet as well as the chemical determinations used, has been previously described (17). The animals were "pair-fed" by groups, *i.e.*, the intake of the control groups was limited each day to the average intake of the experimental groups. After having been on a potassium-deficient regimen for four weeks, Groups B and C were given Purina® Laboratory Chow and distilled water *ad libitum* for the next six to seven months. The final average weight of the experimentals was 460 Gm. while the final average weight of the controls was 485 Gm.

Bacteriological technique. The strain of *E. coli* used for intravenous inoculation had been isolated from the urine of a patient with active pyelonephritis, identified, injected intravenously into a rat with a ligated ureter, subsequently recovered from the infected kidney, and inoculated into tubes containing 10 ml. of sterile broth. The tubes were incubated for three hours, sealed with parafilm, frozen and stored at −20° C. Colony counts had been plotted against Klett-Summerson colorimeter readings on a broth culture at frequent intervals in order to simplify the determination and standardization of inoculum size for future experiments. A few days before the rats of Group C were to be injected with *E. coli*, a frozen culture was warmed to 37° C., incubated for 12 hours, and a small inoculum placed in 150 ml. of sterile broth. When growth produced marked turbidity, the culture was diluted with sterile broth to the desired colorimeter reading, *i.e.*, equivalent to the number of million bacteria injected. Then 20 to 25 ml. was removed, centrifuged and the sediment resuspended in an equivalent volume of sterile saline. One ml. of this saline suspension, calculated to contain approximately 100,000,000 *E. coli*, was injected into the tail veins of three rats, each with a ligated ureter. After an interval

of four days, these rats were sacrificed, the organism recovered, identified, incubated in sterile broth for approximately six hours, diluted with sterile broth to the desired colorimeter reading, the organism resuspended in saline, and 1 ml. of this saline suspension injected into the tail veins of the animals of Group C. The actual number of bacteria injected was 105,000,000 by colony count. Further details of bacteriological techniques have been previously described (8).

Experimental design. The experimental design is shown in Table I. Group A was sacrificed after four weeks for documentation of the degree of potassium depletion and of the resultant histological changes. Group B animals were sacrificed six months after potassium *repletion* for analysis of renal histology, potassium content of skeletal muscle and culture for spontaneous infection. The rats of Group C were injected intravenously with *E. coli* seven months after potassium repletion. They were arbitrarily sacrificed on either the eighth or ninth day after injection.

One of the control animals died 24 hours after injection. Since it had been dead for an indefinite time before discovery and since this is an insufficient time for the development of gross or microscopic lesions of pyelonephritis, it was eliminated from the study.

TABLE I

Experimental design

Group	Description	No. of experimental rats	No. of control rats
A	K depletion 4 weeks. Analysis of renal histology, serum electrolytes, muscle K, culture for spontaneous infection.	5	5
B	K depletion 4 weeks. Repletion 6 months. Analysis of renal histology, muscle K, culture for spontaneous infection.	5	5
C	K depletion 4 weeks. Repletion 7 months. 105,000,000 *E. coli* I.V.	14	14

TABLE II

Groups A and B—Analyses of sera and skeletal muscle

	Serum					Muscle	
	Na	K	Cl	CO_2	BUN	H_2O	K
	mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mg./100 ml.*	*Gm./100 Gm. FFDS**	*mEq./100 Gm. FFDS*
Acute K depletion—Group A							
	147.0	2.4	69.8	48.0		327	27.1
	143.0	2.4	83.1	37.4		322	29.4
	145.0	2.8	89.2	37.0		319	27.9
	145.5	2.3	70.8	48.3		313	26.9
	151.5	2.3	67.8	31.7		323	27.4
Controls							
	145.0	4.8	106.4	15.8		334	46.5
	149.5	5.4	104.0	26.0		330	46.2
	145.3	5.1	101.7	22.3		324	45.5
						327	42.5
	143.8	5.8	101.7	21.0		337	46.4
K-depleted with long-term repletion—Group B							
	146.8	4.8	100.5	24.6	26	318	44.4
	142.5	4.6	93.8	28.9	22	318	44.5
	145.2	4.4	100.5	25.0	21	325	46.5
	144.9	4.5	98.5	28.7	23	325	46.2
	143.4	4.1	99.3	23.3	24	332	46.2
Controls							
	143.0	4.6	98.8	20.9	21	313	46.0
	146.0	4.5	100.0	21.6	21	316	47.9
	142.7	4.3	95.3	28.4	24	317	47.4
	147.8	4.8	98.5			320	46.2

* FFDS = fat-free dry solids.

RESULTS

Group A

The results of potassium depletion for four weeks in the animals of Group A are shown in Table II. Analyses of serum electrolytes as compared with their controls show a marked hypokalemic, hypochloremic alkalosis. Serum sodium remained normal. There was a marked decrease in skeletal muscle potassium content when measured in terms of milliequivalents of potassium per 100 Gm. of fat-free dry solids. Histological study of the kidneys of these animals revealed the characteristic cellular alterations in the tubules expected in acute, severe potassium depletion. These tubular lesions,

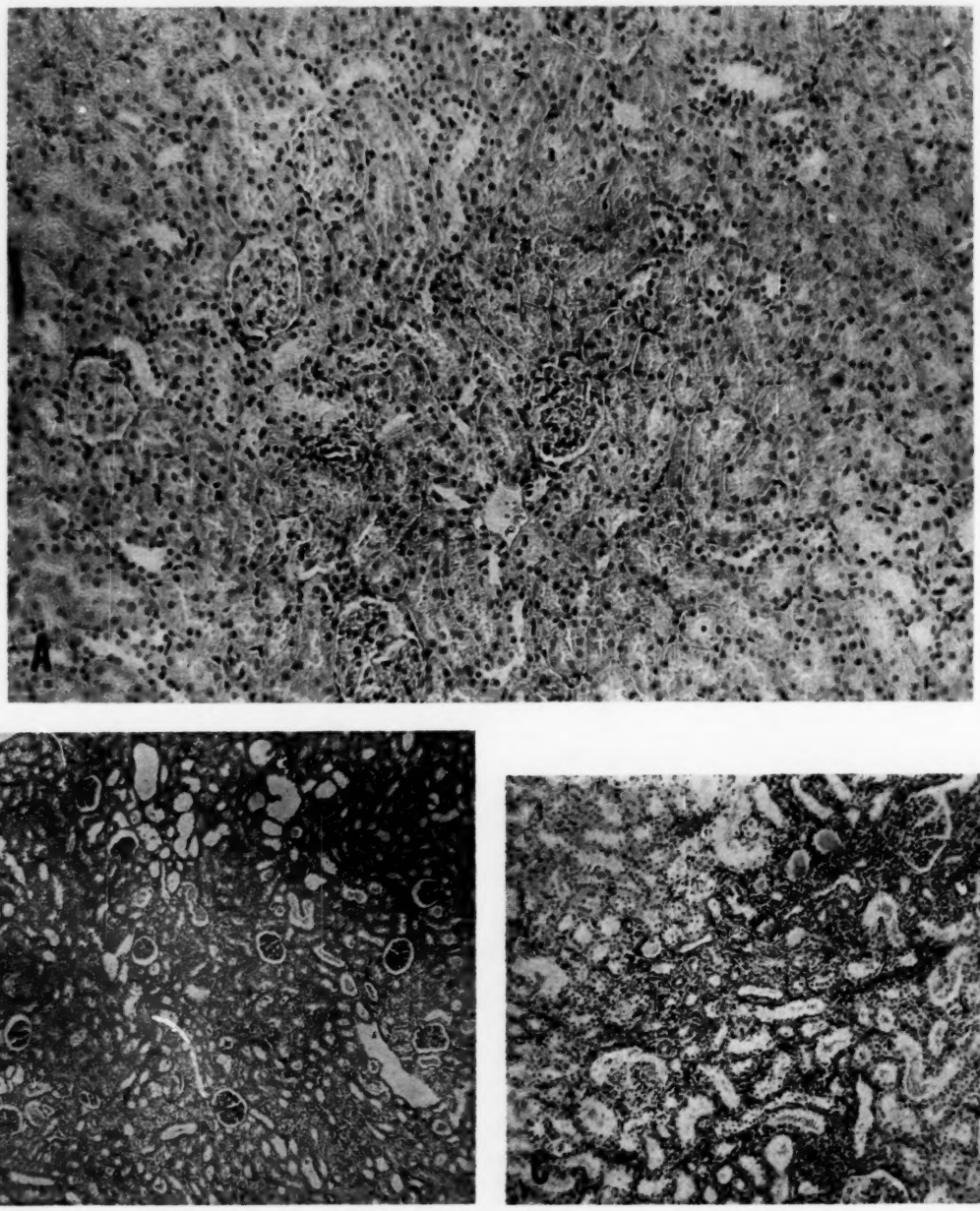

FIG. 1—*Continued*

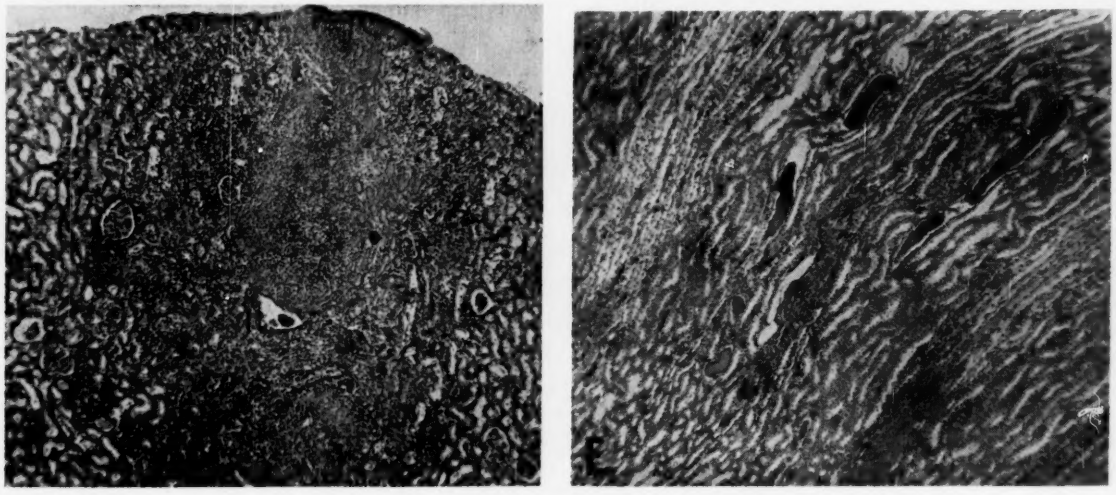

FIG. 1. ILLUSTRATIONS OF NORMAL AND PATHOLOGIC HISTOLOGY ENCOUNTERED

A. Normal rat kidney (×115). **B**. Kidney from a Group A rat after four weeks of potassium depletion. There are many dilated tubules containing homogenous material (×75). **C**. Infiltration of mononuclear cells in the Group C control whose kidneys were sterile on culture (×75). **D**. Renal cortical abscess (×75). **E**. Kidney from a Group B rat. Potassium repletion for six months. Scattered tubular dilatation is present (×75). Hematoxylin and eosin.

the renal lesions after long-term repletion (Group B), and the changes of superimposed pyelonephritis, are illustrated in Figure 1.

Group B

The five rats in this group, which received a stock diet adequate in potassium for six months following a four week period of potassium depletion, had normal serum electrolytes, blood urea nitrogen and muscle potassium (Table II). Their kidneys appeared larger than control kidneys and contained structural abnormalities consisting of dilated tubules containing eosinophilic casts. However, in comparison with the severe residual pathology reported by Fourman, McCance and Parker (12), the lesions were rather minor.

Group C

The 14 experimental rats in this group were treated the same as those in Group B except that the period of potassium repletion was of seven months' duration rather than six, for technical reasons. They were injected with 105,000,000 *E. coli* and sacrificed in eight to nine days. Of the 14, 9 had both cultural and microscopic evidence of pyelonephritis while a tenth had a colony count of 1,920,000 bacteria per Gm. of kidney tissue, but no microscopic evidence of infection (Table III). Seven had *macroscopic* abscesses in one or both kidneys.

Of the 13 controls, 2 had cultural, but no microscopic evidence of pyelonephritis, while a third had microscopic evidence of infection, but the kidneys were sterile on culture.

TABLE III

Group C—Colony count and descriptions of kidneys, after injection of E. coli, of experimental animals seven months after potassium depletion, and of control animals*

Kidney of K-depleted	Description†	Kidney of control	Description†
3,700,000‡	+	0	+
150,000‡	+	0	0
31,000	+++	0	0
970,000	+++	0	0
0	0	1,200,000	0
0	0	0	0
400,000	+++	0	0
23,800	+++	0	0
0	0	0	0
960,000	+++	0	0
100,000	+++	0	0
530,000	+++	0	0
0	0	75,000‡	0
1,920,000	0		

* Inoculum = 105,000,000 organisms.
† 0, no cellular infiltrate; +, cellular infiltrate; ++, microscopic abscesses; +++, macroscopic abscesses.
‡ Lung abscess also due to *E. coli*.

Two of the experimentals and one of the controls also had lung abscesses due to *E. coli*. No other sites of extrarenal infection were found.

In summary, 10 of 14 previously potassium-depleted rats, but only 3 of 13 controls, developed varying degrees of pyelonephritis ($p < 0.05$).[1]

DISCUSSION

There is a convincing body of evidence that obstruction to urine flow, whether in the kidney or in the lower urinary tract, predisposes to infection. The residual damage following an episode of acute potassium depletion is characterized by dilated tubules which are presumably obstructed (13). Therefore, it is not surprising that previously potassium-depleted rats have increased susceptibility to renal infection.

The decision as to whether a given kidney is infected presents no problem when a high colony count of bacteria is combined with gross abscesses or microscopic evidence of pyelonephritis. The decision is quite difficult when there is only a borderline colony count or cellular infiltrate in a kidney which is sterile bacteriologically. In Group C, 7 of 14 repleted rats had *macroscopic* abscesses. Abscesses were present in none of the 13 controls. Two of the repleted rats had kidneys containing heavy infiltrates of mononuclear cells accompanied by colony counts of 3,700,000 and 150,000 *E. coli* per Gm. of kidney. Since cellular infiltration was not seen in uninjected, repleted animals (Group B) and since these are higher colony counts than are to be expected in normal kidneys after this interval (3), they were considered infected. The kidneys of one repleted animal and two controls had counts of 1,920,000, 1,200,000 and 75,000, but no microscopic evidence of pyelonephritis. However, the lesions of pyelonephritis are often quite small and discrete and may therefore be missed when only one or two sections are made from a kidney. Also, since one-half of each kidney was homogenized for culture while the other half was fixed for microscopic study, it was possible to have localized infection in only the half which was cultured. One control animal had a heavy cellular infiltrate, but was sterile on culture. These four animals were considered to be infected, but even if they are considered negative, the overall conclusions are not significantly affected.

The susceptibility of rats to renal infection during *acute* potassium nephropathy was also studied (18), but since the number of animals was small and the results inconclusive, this problem is being made the subject of further investigation.

This study not only lends support to the clinical observations that potassium deficiency predisposes to renal infections (14, 15), but also raises the question of whether an episode of potassium depletion in humans may permanently increase susceptibility to pyelonephritis.

[1] A chi-square test was used to calculate p values.

SUMMARY

Rats with renal lesions resulting from a previous episode of potassium depletion have been shown to exhibit increased susceptibility to experimental pyelonephritis. The factor responsible for this increased susceptibility is unknown, but is most likely tubular obstruction or "internal hydronephrosis."

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THE EFFECT OF PROGESTERONE ON THE RESPIRATION OF PATIENTS WITH EMPHYSEMA AND HYPERCAPNIA *

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The possibility that progesterone could influence breathing was first suggested by studies investigating the respiration of pregnant women. In 1912 Leimdörfer, Novak and Porges (1) found that the alveolar concentration of CO_2 was lower in pregnant women than it was in the same individuals when they were not pregnant. These observations were amplified and confirmed in 1915 by Hasselbalch and Gammeltoft (2). Beginning in 1947, Döring, Heerhaber and co-workers (3–5) investigated the effect of pregnancy in lowering the alveolar CO_2 tension and later extended the studies to measurements of CO_2 tension made during the normal menstrual cycle. They were able to show that the alveolar CO_2 tension was depressed in the luteal phase of the cycle and that if pregnancy occurred this depression continued throughout gestation, rising shortly after delivery. This suggested to them that progesterone might play a role in the genesis of the decrease in alveolar CO_2 tension and they were able to show that 50 mg. of progesterone given intramuscularly to normal men caused a stimulus to breathing which reached its maximum about 12 hours after injection.

Persistent hypercapnia is a frequent finding in the terminal stages of chronic pulmonary disease. Present long-term methods for lowering this hypercapnia are not satisfactory. Since Döring and Heerhaber's studies were limited to single injections of progesterone, and because the effect of progesterone on respiration in the presence of hypercapnia was not known, the following studies were done to investigate the effect of repeated injections of progesterone on the respiration of patients with chronic pulmonary disease and elevated levels of arterial pCO_2. In an attempt to evaluate the relation of chemical structure to function, some analogues of progesterone have also been investigated.

METHODS

Patients were selected whose diagnosis of emphysema was established on the basis of the typical history, physical and radiological signs of chronic obstructive disease of the lungs, plus changes of an elevated arterial CO_2 tension. All patients continued to have significant respiratory symptoms in spite of intensive bronchodilator therapy. They had been in the hospital at least three weeks before the first control values were obtained and during this period had reached their maximum response to ordinary therapeutic measures as judged by the stability of their clinical condition and by measurements of timed vital capacity and maximum breathing capacity. Thus they were in a stable enough condition to allow demonstration of the hormonal effects on the respiration without the necessity of other changes in their therapy.

The subjects were studied when they were lying in the supine position, approximately two hours after a light breakfast. No special attempt was made to have them completely basal, but they were all thoroughly familiar with the procedure and it is believed that anxiety was minimal. An arterial needle was introduced into a brachial artery, after which a five minute rest period was allowed for the patients to become stable. Their expired gas was then collected over a three minute period and, during the second minute, arterial blood samples were taken. The arterial pH was determined immediately in a Cambridge Model R pH meter with a constant temperature electrode set at 37° C. Simultaneous samples agreed within 0.02 pH unit. Whole blood CO_2 content was determined according to the method of Van Slyke and Neill (6) and the pCO_2 was determined by the nomograph of Singer and Hastings (7). The expired gas was analyzed for oxygen and carbon dioxide with the Scholander microanalyzer. Duplicates agreed within 0.04 per cent. The Bohr equation was used to calculate the physiological dead space, and the alveolar ventilation was estimated by the equation $\dot{V}_A = f\ (V_T - V_D)$.[1] Estimates of total vital capacity with one and two second fractions were carried out with a Collins respirometer, using standard methods.

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[1] $\dot{V}_A$ = alveolar ventilation per minute, f = respiratory frequency, V_T = tidal volume and V_D = physiological dead space volume.

In some instances, arterial pCO_2 was determined by the rebreathing method of Hackney, Sears and Collier (8). In 22 simultaneous measurements (range 40 to 78 mm. Hg) the mean difference between the directly measured arterial pCO_2 and the rebreathed value was -0.4 ± 0.4 (± 1 standard error).

RESULTS

The vital capacity and the one and two second fractions obtained on each patient are shown in Table I. The values for ventilation, pCO_2 and gas exchange during the study periods in the nine patients are given in Table II. The mean changes from pretreatment, and post-treatment controls brought about by progesterone,[2] 50 mg. daily, intramuscularly, are shown in Table III. The mean values were calculated by averaging the values from each period—pretreatment control, treatment, or post-treatment control—to obtain a value representative of this period for each patient. These mean values were then averaged to give the mean for all the subjects. The mean arterial carbon dioxide tension (Pa_{CO_2}) declined by 8.4 mm. Hg during treatment, followed by a rise of 5.9 mm. Hg when the drug was discontinued. Associated with the decrease in pCO_2 there was a rise in minute ventilation and alveolar ventilation of 1.78 and 0.75 L. per minute, respectively. The post-treatment control showed a 1.32 L. per minute fall in minute ventilation and a fall of 0.79 L. per minute in alveolar ventilation. Mean pH rose by 0.024 unit with progesterone and declined by 0.024 unit when the drug was discontinued. All these changes are statistically significant. The values obtained in Subject 1 are shown in Figure 1. Measurements during the first two days of treatment showed an effect by the thirtieth hour, but this was not maximal, for the Pa_{CO_2} fell 6 more mm. Hg during the next six days of treatment.

Anhydrohydroxyprogesterone[3] was given to Subjects 2 and 8. Complete studies were carried out in Subject 2 and there was no significant rise in ventilation, or fall in pCO_2. The findings in Patient 8 are shown in Figure 2. Complete studies could not be carried out in this individual because of the severity of his illness, but during the administration of anhydrohydroxyprogesterone, his arterial pCO_2 steadily rose as his clinical condition deteriorated; when progesterone was substituted he had a prompt fall in pCO_2 from 63 to 44 mm. Hg, and this was maintained with 50 mg. of progesterone given intramuscularly every third day. No other change in therapy was made during the period of study. This patient was confused and had a coarse tremor during the period of marked Pa_{CO_2} elevation. These findings disappeared when the hypercapnia was lowered by progesterone. He subsequently expired and extensive emphysema was found on postmortem examination.

Subject 3, as shown in Figure 3, had a fall in Pa_{CO_2} of 10 mm. Hg on progesterone, and when 1.8 Gm. aspirin daily was added to this, a further rise in minute ventilation and alveolar ventilation was produced. However, pCO_2 declined only 2 mm. Hg and oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) rose significantly. This patient was also given oral Progesterone Linguets®, 50 mg. per day. There was no effect on ventilation. Subsequent administration of intramuscular progesterone produced a fall in pCO_2.

Subjects 4 and 7 received a placebo intramuscularly during the control period. This produced no change in ventilation and both patients subsequently responded to intramuscular progesterone.

In Subject 9, 19 nor-ethinyl-testosterone produced no change in ventilation or pCO_2. This patient subsequently responded to progesterone.

1,2-Dehydroprogesterone was given to Patients 4 and 7, and in both instances it failed to produce

TABLE I

Timed vital capacity measurements

Patient no.	Total vital capacity	% Predicted normal values	1 Second vital capacity	2 Second vital capacity
	L.		*L.*	*L.*
1	1.22	46	0.50	0.77
2	1.79	58	0.54	0.81
3	1.82	48	0.50	0.77
4	3.28	88	1.21	1.93
5	2.06	54	0.76	1.16
6	2.16	65	0.58	0.81
7	0.98	37	0.49	0.80
8	1.75	46	0.45	0.72
9	2.46	62	0.67	1.07

[2] All steroid compounds and placebo kindly supplied by Dr. Henderson of the Schering Corp., Bloomfield, N. J.

[3] 17 Ethinyl-testosterone.

TABLE II

Changes in ventilation and gas exchange produced by hormone treatment

Patient no.	Age	Sex		$\dot{V}_E$*	Pa_{CO_2}	$\dot{V}_A$	$\dot{V}_{O_2}$	$\dot{V}_{CO_2}$	R*	pH
			Date	*L./min.*	*mm. Hg*	*L./min.*	*cc./min.*	*cc./min.*		
1	59	M	1/1	4.28	61	1.56	145	112	0.77	7.34
			1/18	4.54	61	1.82	157	130	0.83	7.38
			Progesterone 50 mg. I. M.							
			(24 hr.) 1/19	6.12	59	2.39	191	164	0.86	7.35
			(30 hr.)	6.34	57	2.53	207	170	0.82	7.37
			1/23	6.98			199	182	0.91	
			1/26	6.96	51	2.99	189	176	0.93	7.41
			Progesterone discontinued							
			1/31	6.22	61	2.42	189	171	0.90	7.36
			Progesterone 50 mg. I. M.							
			2/7	5.74	54	2.47	167	157	0.94	7.38
2	79	M	6/1	10.14	58	3.10	286	255	0.90	7.38
			6/6	10.21	59	3.58	276	243	0.88	7.37
			Anhydrohydroxyprogesterone 50 mg. q. i. d.							
			6/20	10.48	55	3.45	250	218	0.87	7.38
			Progesterone 50 mg. I. M.							
			6/29	11.85	51	4.15	277	249	0.90	7.37
			Progesterone discontinued							
			7/5	8.02	59	2.64	219	182	0.83	7.35
3	59	M	4/18	7.74	54	3.94	295	248	0.84	7.38
			4/23	9.56	54	3.83	248	243	0.98	7.35
			4/26	8.94	54	3.40	222	211	0.95	7.38
			Progesterone 50 mg. I. M.							
			5/2	10.4	44	4.06	224	208	0.93	7.40
			5/7	8.74	44	4.02	219	207	0.95	7.39
			Progesterone 50 mg. I. M. and aspirin 1.8 Gm.							
			5/11	12.11	42	5.21	257	253	0.98	7.35
			Progesterone and aspirin discontinued							
			5/16	8.83	51	3.44	226	207	0.92	7.34
			Oral progesterone 10 mg. 5 times daily sublingually							
			5/23	7.53	61	2.94	227	209	0.95	7.35
			5/31	8.03	51	3.13	218	193	0.89	7.37
4	62	M	9/11	10.52	55	4.21	317	276	0.87	7.32
			9/16	11.00	53	4.92	352	305	0.86	7.34
			Progesterone 50 mg. I. M. (9/17 to 10/1)							
			10/1	16.20	49	6.97	384	394	1.03	7.33
			Placebo 10/3 to 10/10							
			10/8	15.17	54	5.62	370	356	0.96	7.31
			1,2 Dehydroprogesterone 25 mg. I. M. daily (10/10 to 10/16)							
			10/16	14.14	50	4.89	314	274	0.87	7.36
			10/24	16.40	51	5.58	325	331	1.01	7.36
5	59	M	3/1	8.52	46	3.07	206	163	0.79	7.39
			3/5	11.0	45	4.07	254	216	0.85	7.39
			Progesterone 50 mg. I. M.							
			3/12	11.9	41	4.76	260	223	0.86	7.41

TABLE II (*Continued*)

Patient no.	Age	Sex		$\dot{V}_E$*	Pa_{CO_2}	V_A	$\dot{V}_{O_2}$	$\dot{V}_{CO_2}$	R*	pH
			Date	*L./min.*	*mm. Hg*	*L./min.*	*cc./min.*	*cc./min.*		
			Progesterone discontinued							
			4/5	10.65	46	3.72	248	201	0.81	7.39
			4/11	10.80	45	3.88	253	247	0.98	7.39
6	74	M	2/29	8.93	55	2.05	202	161	0.80	7.35
			3/2	7.82	55	2.27	198	149	0.75	7.36
			Progesterone, 50 mg. I. M.							
			3/8	8.70	42	3.04	183	150	0.82	7.43
			Progesterone discontinued							
			3/23	8.01	49	2.57	175	148	0.85	7.41
7	63	F	12/19	9.39	52	3.48	257	208	0.81	7.39
			12/31	9.72	53†	3.69	197	214	1.09	
			3/4	5.95	53	2.21	206	137	0.67	7.40
			Placebo 3/5 to 3/18							
			3/11		52†					
			3/18	8.36	51	2.93	210	172	0.82	7.40
			Dehydroprogesterone 25 mg. I. M. (3/18 to 3/24)							
			3/24	9.41	52	2.08	230	181	0.79	7.39
			Progesterone 50 mg. I. M. (3/24 to 4/2)							
			3/31	10.09	47	3.53	234	195	0.83	7.37
			4/9	8.99	47	3.43	213	178	0.84	7.39
8	60	M	12/31		51					7.43
			1/2		51					7.43
			1/3		Anhydrohydroxyprogesterone 50 mg. q. i. d. to 1/11					
			1/8		55					7.42
			1/11		59					7.44
			1/16		63					7.40
			1/19		Progesterone 50 mg. I. M. daily					
			1/22		45					7.45
			1/25		Progesterone 50 mg. I. M. every other day					
			1/29		46					7.48
			2/5		45					7.42
			2/6		43					7.50
			2/8		Progesterone 50 mg. I. M. every third day					
			2/13		42					7.48
9	49	M	4/2	12.04	50	3.98	263	233	0.89	7.37
			4/3	10.72	52	3.11	226	187	0.83	7.36
			19 Nor-ethinyl-testosterone 10 mg. orally t. i. d.							
			4/5	11.07	54	3.40	236	204	0.86	7.37
			Progesterone 50 mg. I. M. daily							
			4/9	12.26	47	3.94	227	218	0.96	7.39

* $\dot{V}_E$ = volume of gas expired per minute; R = respiratory exchange ratio.
† Rebreathed method for Pa_{CO_2}.

an increase in ventilation, but in Patient 4 there was a 4 mm. fall in pCO_2. However, in this case the post-treatment control period failed to show a rise.

DISCUSSION

It is apparent from the data that progesterone is capable of lowering the arterial pCO_2 in patients with emphysema and hypercapnia. That this hormone lowers the alveolar pCO_2 in normal subjects has been demonstrated by several observers in the past. Döring, Loeschcke and Ochwadt (5) gave 50 mg. of progesterone intramuscularly to five normal subjects and tested its effect on the CO_2 stimulus to respiration. Twenty-four hours after injection there was a drop of

TABLE III

Mean changes from control values produced by progesterone

	Progesterone 50 mg. I.M. daily					
	Change from control period during treatment		No. of observations	Change during after-control period		No. of observations
Pa_{CO_2} (*mm. Hg*)	−8.4 ± 1.4 (± 1 S.E.)	$p < 0.01$	10	+5.9 ± 1.3	$p < 0.01$	7
$\dot{V}_E$ (*L./min.*)	+1.78 ± 0.56	$p < 0.02$	9	−1.32 ± 0.42	$p < 0.02$	7
$\dot{V}_A$ (*L./min.*)	+0.76 ± 0.24	$p < 0.02$	9	−0.79 ± 0.19	$p < 0.01$	7
pH	+0.024 ± 0.0096	$p < 0.05$	10	−0.024 ± 0.0097	$p < 0.05$	7

2.18 mm. pCO_2 from control values, both measurements being made at the point in the CO_2 stimulus curve when the patient was breathing 12 L. of the CO_2 mixture per minute. Estradiol did not cause a lowering of the resting pCO_2. Progesterone caused a small rise in body temperature, but a greater increase in temperature induced by a pyrogen did not alter the pCO_2. Their conclusion was that progesterone produced a primary stimulus to respiration and did not cause the hyperventilation by bringing about a metabolic acidosis. Essentially the same results were found by Goodland, Reynolds, Pommerenke and McCoord (9, 10).

If one assumes that the action of progesterone causes a small but definite degree of hyperventilation, then it is of interest that this increase in ventilation is sufficient to cause a fall in Pa_{CO_2} in patients with severe obstructive disease and hypercapnia. It has been shown by Wilson, Borden,

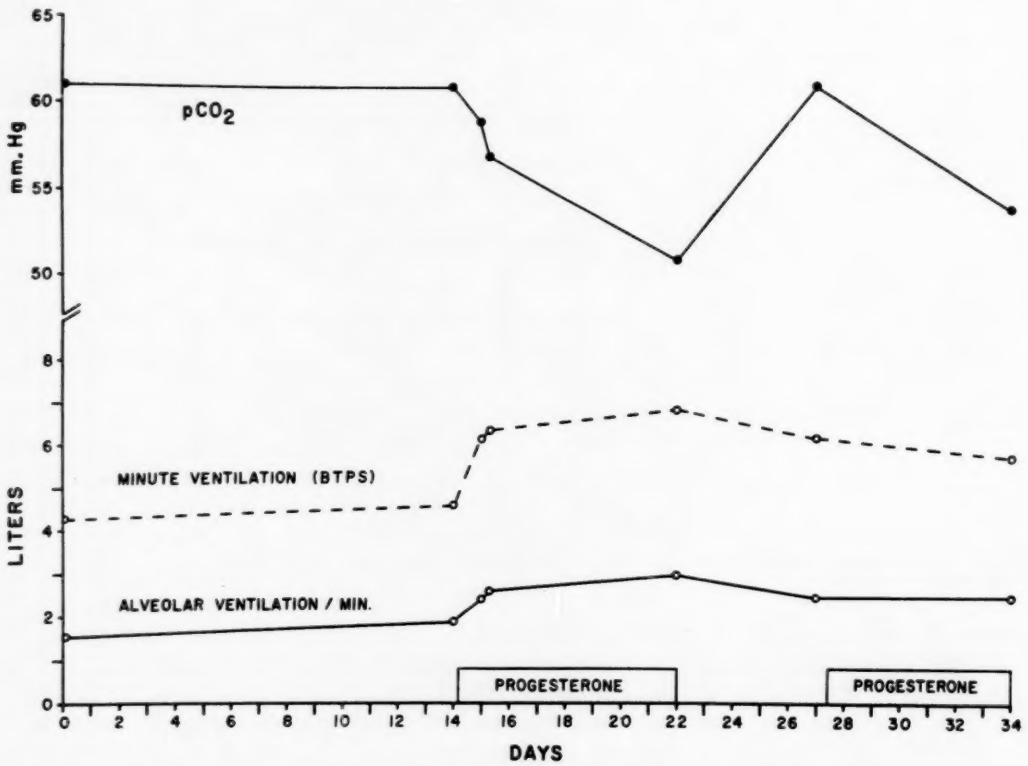

FIG. 1. VALUES FOR ARTERIAL CO_2 TENSION, MINUTE VENTILATION AND ALVEOLAR VENTILATION DURING THE STUDY PERIOD IN PATIENT 1

Progesterone, 50 mg. per day, was given intramuscularly during the indicated periods.

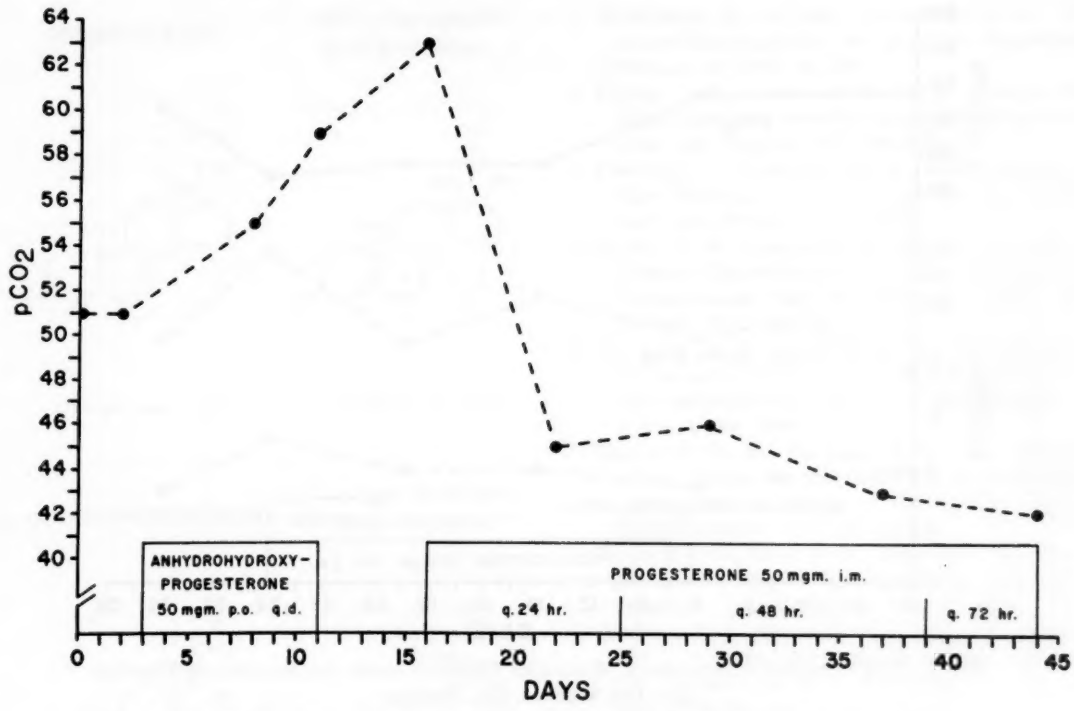

FIG. 2. THE EFFECT OF PROGESTERONE ON INCREASING HYPERCAPNIA IN PATIENT 8

Without other change in therapy, progesterone produced a lowering in arterial CO_2 tension; anhydrohydroxyprogesterone was ineffective.

Ebert and Wells (11) that voluntary hyperventilation is incapable of lowering the Pa_{CO_2} in patients with emphysema and hypercapnia.

On the basis of a theoretical analysis, Otis (12) has related the alveolar CO_2 tension to the work of breathing and the minute ventilation. As the ventilation increases, a point is reached when it is no longer effective in lowering the alveolar CO_2 tension because the increased volume of CO_2 produced by the increased work of breathing cannot be eliminated by the ventilation. With the increased work of breathing that exists in emphysema, this point at which ventilation ceases to be effective in lowering CO_2 tension is shifted to lower values than in the normal subject. Presumably this point would vary with the degree of emphysema. Since, in the present study, the arterial CO_2 tension fell and the arterial pH increased, progesterone caused an increase in effective ventilation.

Aspirin added to progesterone in Patient 3, as shown in Figure 3, caused a marked further augmentation in ventilation over and above the increase in resting level produced by the hormone alone, but brought about only a 2 mm. Hg fall in Pa_{CO_2}. However, aspirin caused a sharp rise in CO_2 production and this may have canceled the effect of hyperventilation in so far as lowering the arterial CO_2 tension was concerned. The ability of aspirin to lower the Pa_{CO_2} in hypercapnia has been claimed by some (13) and disputed by others (14). It is of interest that in another study in this laboratory, on individuals without heart or lung disease, progesterone produced approximately the same decline in alveolar CO_2 tension as did aspirin, 2 Gm. every six hours (15).

Riley (16) has reviewed the problem of hypercapnia and the increased work of breathing, and has concluded that the hypercapnia may be a desirable adjustment in that it enables the patient to eliminate carbon dioxide at a lower minute ventilation and, therefore, at a smaller work of breathing than would be possible if the ventilation were driven at a high enough level to main-

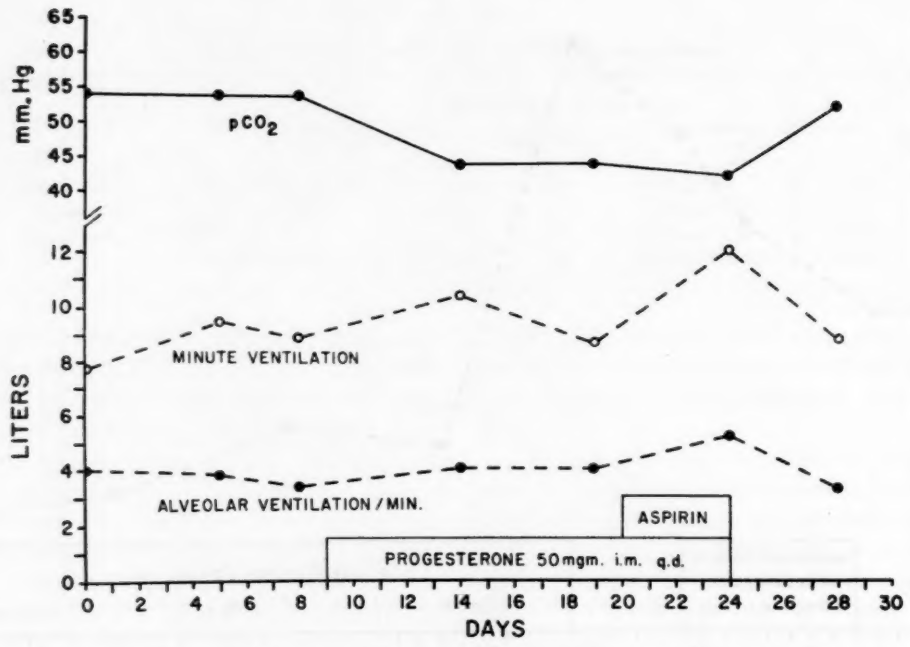

FIG. 3. THE EFFECT OF PROGESTERONE, WITH AND WITHOUT ADDED ASPIRIN, ON THE VENTILATION AND ARTERIAL CO_2 TENSION

When 1.8 Gm. aspirin per day was added, there was a marked increase in ventilation but only a small decrease in CO_2 tension.

tain the CO_2 tension in the normal range. This adjustment to hypercapnia reduces the oxygen consumption necessary for ventilation and leaves more for nonventilatory work. Riley thus stresses the value of reducing the work of breathing to lower the hypercapnia associated with emphysema. However, this is an adjustment which the patient makes at some detriment to his overall condition because of the well known cerebral depressant effects caused by an elevated arterial CO_2 tension.

On the basis of preliminary studies it would appear that the effect of progesterone on ventilation is not solely the result of progestational activity, because anhydrohydroxyprogesterone and 19 nor-ethinyl-testosterone, both of which were employed in doses known to have marked progestational activity, were ineffective. The structural formulas of the hormonal compounds employed are given in Figure 4. Apparently the ethinyl group inactivates the respiratory effects seen with progesterone itself. Also, it appears that alterations of the molecular structure at other sites can abolish the effect; *e.g.,* substituting a double bond in the 1, 2 position. However, more subjects should be studied with these three compounds before a definite statement can be made as to their activity.

Conclusions on the exact mode of action of progesterone are not possible from this study. Robin, Travis and Crump (17) have found that, in five normal subjects, progesterone lowered the arterial pCO_2 without altering the ventilatory response to carbon dioxide, and inspection of the CO_2 stimulus curves of Döring, Loeschcke and Ochwadt (5) failed to show an increase in responsiveness with progesterone. It is felt that other areas of the brain, possibly the hypothalamus, may represent the site of action.

It appears that progesterone is effective in lowering the Pa_{CO_2} in patients with emphysema and hypercapnia. In one patient the effect was striking. However, this study does not establish its clinical usefulness in individuals with severe elevations of pCO_2.

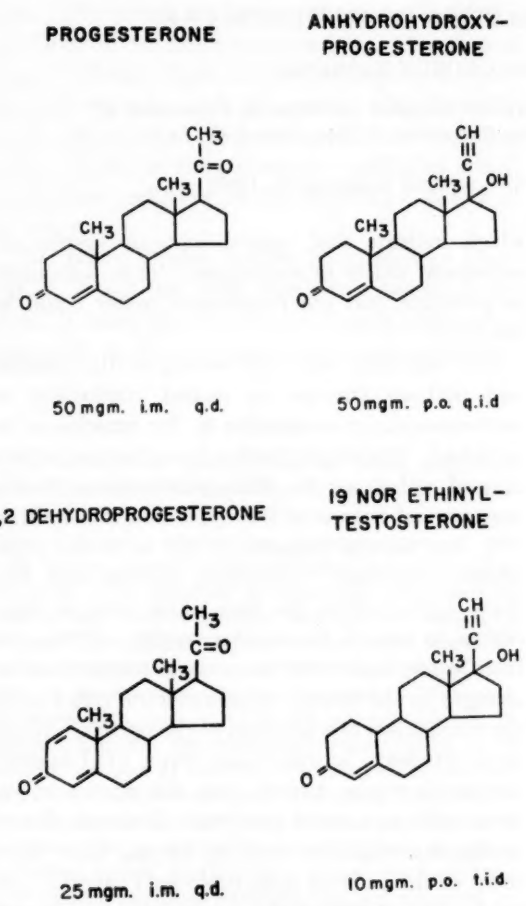

FIG. 4. THE STRUCTURAL FORMULAS OF THE COMPOUNDS EMPLOYED

SUMMARY

1. Progesterone produces hyperventilation and lowers the arterial pCO_2 in patients with emphysema and stabilized hypercapnia.

2. Limited studies with compounds other than progesterone suggest that this progestational property is not sufficient in itself to cause the hyperventilation effect.

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THE PULMONARY ABNORMALITIES IN MYXEDEMA * †

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The purpose of this paper is to report a study of lung function in patients with myxedema. Recently we studied 28 obese persons and found 10 who had alveolar hypoventilation manifested by increased arterial P_{CO_2} (2). They had either lung disease or myxedema in association with their obesity. The nature of pulmonary involvement in patients with myxedema has not been studied. Why did patients with obesity and myxedema develop alveolar hypoventilation? We postulated that patients with myxedema might have one or all of the following: a depression of the respiratory center in the brain, interference with neural conduction or with neuromuscular transmission to the respiratory muscles, disease of the respiratory muscles, or a change in the character of the alveolar capillary membrane.

The existence of central nervous system abnormalities in myxedema is known. In 1904, Gull (3) included myxedema under "Diseases of the Nervous System" in a collection of his writings. Scheinberg, Stead, Brannon and Warren (4) measured cerebral blood flow in eight patients with myxedema by the nitrous oxide technique and found that the average cerebral blood flow was 38 per cent below normal, oxygen and glucose consumption 27 per cent below normal, and cerebral vascular resistance 91 per cent above normal. Browning, Atkins and Weiner (5) found encephalographic changes in seven psychotic adults with myxedema. The electroencephalogram returned toward normal as the patient's myxedema improved. These are a few of the many studies which indicate that central nervous system involvement exists in myxedema. It is reasonable to postulate that the respiratory center could be involved.

The fact that there are lesions in the muscles and perhaps changes in neural conduction or neuromuscular transmission to the muscles is established. Pathologic studies have demonstrated a mucoid substance in skin, subcutaneous tissue, mucous membranes of the upper respiratory tract (6), and skeletal muscles (7–9) in severe myxedema. Lambert, Underdahl, Beckett and Mederos (10) believe the slow ankle jerk in myxedema is caused by an abnormality of the contractile mechanism of the muscle rather than by changes in the neural elements of the reflex or in the mechanism of excitation of the muscle. Waldstein, Bronsky, Schrifter and Oester (11) inserted needle electrodes directly into the muscle of patients with myxedema and found abnormal electromyograms which reverted to normal after treatment of the patients with desiccated thyroid. Ingold (12) abolished the prolonged muscle contraction and relaxation time in myxedematous rats by blocking transmission at the neuromuscular junction with curare. Whether the primary lesion involves the muscle, neural conduction, or neuromuscular transmission is beyond the scope of this paper. "Muscular" involvement might be expected to produce changes in the patient's ability to ventilate his lungs.

Evidence of capillary involvement in myxedema is available. Zondek, Michael and Kaatz (13) studied the ungual limbus capillaries in six patients with myxedema and found them to be reduced in number and size. After thyroid therapy the capillaries were present in normal number and the caliber of the vessels was normal. Lange (14) studied five patients with myxedema and found a large increase in capillary permeability which returned to normal following treatment of the myxedema. In autopsy material, Baker and Ham-

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ilton (15) found thickening of the walls of the capillaries of the heart in myxedema. Foster and Barr (7) found slight but definite fibrous thickening of the alveolar walls of a patient who died with severe myxedema. It is reasonable to postulate similar lesions in the pulmonary capillaries which might cause reduction in the diffusing capacity of the lungs.

This paper reports studies of pulmonary function in 26 patients with myxedema before treatment. Twenty-one were studied again after treatment with desiccated thyroid or triiodothyronine.

METHODS

The patients were selected from the wards and clinics of the University Hospitals and Veterans Administration Hospital, Iowa City. Each patient had a complete history, physical examination, roentgenogram of the chest, thyroid function studies and other indicated laboratory tests. The diagnosis of myxedema was based on characteristic history, physical findings and laboratory findings. We required that the protein-bound iodine determination be below 3.0 μg. per 100 ml. of plasma (the range of normal values in the biochemistry laboratory, University Hospitals, is 3.5 to 8.0 μg. per 100 ml. of plasma); and that the radioactive iodine uptake at 24 hours be less than 10 per cent (the range of normal values in the radiation laboratory, University Hospitals, is 15 to 45 per cent). Pulmonary disease was excluded if the patient had no symptoms of lung disease, no physical findings of lung disease, and a normal roentgenogram of the chest. The basal metabolic rate was measured by standard techniques using the Sanborn basal metabolism apparatus. The protein-bound iodine determinations were performed by a slight modification of Barker, Humphrey and Soley's method (16). The 24 hour uptake of radioactive iodine was measured in the Radiation Research Laboratory by the method of Evans (17). The fasting total serum cholesterol was determined by the method of Pearson, Stern and McGavack (18). Thyroid stimulating hormone (TSH) stimulation tests were done when indicated to help differentiate primary from secondary myxedema. Pulmonary function tests and arterial blood studies were done in the morning after the patient had eaten breakfast, according to methods previously described from this laboratory (2). Predicted normal values for vital capacity were calculated on the basis of the patient's height (19). Predicted normal values for inspiratory capacity are 75 per cent of predicted vital capacity; predicted normal values for expiratory reserve volume are 25 per cent of predicted vital capacity. Predicted normal values for total lung capacity were calculated as follows: for patients 15 to 34 years in age, the predicted vital capacity divided by 0.80 (20); for patients 35 to 50 years in age, the predicted vital capacity divided by 0.766 (20); and for patients over 50 years in age, the predicted vital capacity plus 2,430 ml. (21). The predicted normal values for the maximal breathing capacity (MBC) were the mean values found by Baldwin, Cournand and Richards (22) taking into consideration sex and age of the subject but not body surface area. The predicted normal values for the diffusing capacity of the lung for carbon monoxide (D_{Lco}) were based on the regression equation of Ogilvie, Forster, Blakemore and Morton (23) which is D_{Lco} = height (in inches) × 0.874 − 31.6.

Six of our patients were extremely obese and had myxedema. After the initial studies were completed, these were given an 800 calorie diet and desiccated thyroid or triiodothyronine. When possible, patients were hospitalized until they were nearly euthyroid and had lost 50 to 100 pounds. Sometimes this took six months. Four patients had clinical evidence of lung disease in addition to myxedema.

The remaining 16 patients had myxedema, but no clinical evidence of lung disease. These patients were usually hospitalized for "initial" pulmonary studies and the initiation of thyroid therapy. Then they were seen at three month intervals in the medical out-patient department. At the return visit, each patient had an interval history, physical examination and the necessary thyroid function tests. Most of the patients in this group were judged to be euthyroid or nearly so by the time of the last study.

Statistical analysis of the data was done using the test of significant difference in paired data by the method of Fisher (24). The formula used was:

$$t_{(n-1)} = \frac{\bar{d}}{\sqrt{\frac{\Sigma (d - \bar{d})^2}{n (n - 1)}}}$$

where $\bar{d}$ = the difference between the means, $\Sigma (d - \bar{d})$ = sum of the squares of the individual differences minus the difference between the means and n = number of patients studied.

The patients are divided into two groups. Sixteen patients had myxedema but no evidence of lung disease and are considered in Group I. Patients in Group II had myxedema and either (A) were obese (six patients) or (B) had lung disease (four patients).

RESULTS

A. Patients with myxedema but no clinical lung disease

This group consists of 16 patients whose age range was 26 to 68 years. There were 6 men and 10 women. The average weight of the group was 161 pounds. The results of thyroid function studies in these patients are listed in Table I. The mean basal metabolic rate was minus 27; mean protein-bound iodine was 2.0 μg. per 100 ml. of plasma; mean 24 hour radioactive iodine uptake was 3.3 per cent; mean total cholesterol was 399

mg. per 100 ml. of serum. The type of myxedema, the duration of symptoms and the daily dose of desiccated thyroid or triiodothyronine are shown in Table I. All but one of the patients (M. M.) were studied before thyroid treatment was started. The results of pulmonary function studies in these patients are listed in Tables II and III. The lung volumes were normal. Distribution of inspired air was slightly uneven in six patients as measured by the single breath nitrogen test, but normal as measured by the less sensitive test based on the per cent of nitrogen at the end of seven minutes of oxygen breathing. There were slight abnormalities in the mechanical tests. The mean maximal breathing capacity was 82 per cent of predicted normal. The maximal expiratory flow rate averaged 224 L. per minute while the maximal inspiratory flow rate averaged 154 L. per minute. The mean $D_{L_{CO}}$ was definitely reduced, being 68 per cent of predicted normal.

Thirteen patients in this group were studied after approaching or reaching the euthyroid state. The diuresis produced by therapy caused a significant mean weight loss of 14 pounds ($p < 0.01$). The mean basal metabolic rate increased significantly from -28 to -8 ($p < 0.01$); the mean protein-bound iodine increased significantly from 1.9 to 5.9 μg. per 100 ml. of plasma ($p < 0.01$); while the total cholesterol decreased significantly from 431 to 249 mg. per 100 ml. of serum ($p < 0.001$). Treatment did not alter the lung volumes significantly. Mean alveolar ventilation increased slightly from 4.0 to 4.6 L. ($p > 0.2$). In response to breathing 7.5 per cent CO_2 in air, the mean minute volume of ventilation in these 10 patients increased from 18.7 to 21.6 L. ($p > 0.3$). Alveolar gas distribution as measured by the single breath nitrogen test did not change significantly ($p > 0.9$). The maximal breathing capacity increased significantly from a mean of 78 to 102 per cent of predicted normal ($p = < 0.01$). The mean expiratory flow rate was 213 L. per minute before thyroid replacement and 250 L. per minute afterward ($p > 0.1$). The maximal inspiratory flow rate increased from a mean of 152 to 167 L. per minute ($p > 0.4$). The $D_{L_{CO}}$ in 12 patients increased significantly from a mean of 69 to a mean of 93 per cent of predicted normal ($p < 0.01$).

The results of arterial blood studies in these patients are listed in Table IV. Mean arterial oxygen saturation at rest was 97 per cent, mean pCO_2 was 39 mm. Hg, mean pH was 7.41, and the mean hematocrit was 35 per cent. Thyroid administration to these patients produced no significant changes except that the mean hematocrit increased from 35 to 40 per cent ($p = < 0.05$).

B. Patients with myxedema and obesity

This group is composed of six patients, five women and one man, with myxedema, whose mean weight was 294 pounds. Their ages ranged from 41 to 67 years. The initials, physical characteristics, results of thyroid function tests, type of myxedema, duration of symptoms and daily dose of thyroid in these patients are listed in Table V. The mean basal metabolic rate was minus 17; the mean protein-bound iodine was 1.6 μg. per 100 ml. of serum; the mean 24 hour uptake of radioactive iodine was 2.8 per cent; the mean total cholesterol was 461 mg. per 100 ml. of serum. Five had primary myxedema and one had myxedema following radioactive iodine therapy for thyrotoxicosis. The average duration of symptoms in this group was 8.5 years. Two of the patients (H. K. and E. K.) received small doses of desiccated thyroid before the initial studies were done. Four patients were restudied after treatment with a reducing diet and desiccated thyroid or triiodothyronine. The mean initial weight in these four patients was 292 pounds and at the time of last study it was 223 pounds, a mean loss of 69 pounds.

The results of lung volume studies in these patients are listed in the top half of Table VI. Initially, the lung volumes were reduced. Mean inspiratory capacity was 76 per cent of predicted normal; mean expiratory reserve volume was 64 per cent of predicted normal; mean vital capacity was 71 per cent of predicted normal and mean total lung capacity was 66 per cent of predicted normal. In the four patients studied after therapy, the lung volumes returned to or toward normal.

In the top half of Table VII, the results of ventilatory studies, respiratory mechanics and diffusion are listed. Initially, in these six patients with myxedema and obesity, the mean minute volume of ventilation was 6.4 L. and the mean alveolar ventilation was 2.9 L. The mean maximal breath-

TABLE I

Physical characteristics, thyroid function tests and treatment in patients who have myxedema but no lung disease

Patient	Age	Sex	Ht.	Wt.	Surface area	Date of study	Basal metabolic rate	Protein-bound iodine	Radioactive iodine uptake (24 hr.)	Total cholesterol	Type of myxedema	Duration of symptoms	Daily dose of thyroid
			in.	*lbs.*	*M.²*			*μg./100 ml. plasma*	*%*	*mg./100 ml. serum*		*yrs.*	*mg.*
V. C.	43	F	65	136	1.67	20 June 58	−26	1.6	3	477	Primary	6	None
				115	1.55	15 Sept. 58	− 7	6.4		227			150
L. A.	62	F	63	131	1.62	23 June 58		1.5	3	426	Primary	10	None
				122	1.55	23 Dec. 58	+ 6	5.3		268			60
R. T.	41	M	71	157	1.90	27 June 58	−38	2.2	0	358	Primary	4	None
				148	1.88	8 Oct. 58	−19	1.3		213			100*
M. Mc.	68	F	67	181	1.91	23 Jan. 58	−21	2.7	3	373	Primary	4	None
				165	1.81	7 Oct. 58		3.6		286			60
H. H.	36	F	65	167	1.83	18 Sept. 58	−28	2.7	4	467	I^{131}	1	None
				167	1.83	19 Feb. 59	−12	5.7		295			60
M. M.	68	F	61	167	1.74	10 Dec. 57	−20	1.9	6	507	Primary	8	30
				154	1.72	27 Mar. 58							120
W. P.	26	M	61	203	1.90	16 Dec. 57	−44	0.9	0	347	Primary	11	None
				203	1.90	23 Apr. 58							150*
				178	1.80	1 Oct. 58	−18	1.1		401			None
				151	1.67	10 Dec. 58	+ 2	9.3		157			150
M. R.	54	F	63	119	1.54	27 May 58	−29	1.2	3	400	Primary	5	None
				111	1.50	30 Sept. 58	+ 1	3.7		270			60
A. C.	47	F	60	163	1.71	14 July 58	−25	1.2	4	347	Post	9	None
				162	1.69	18 Feb. 59	− 8	6.7		238	thyroidectomy		120
G. D.	47	F	65	142	1.70	17 July 58		1.2	4	407	Post	4	None
				142	1.70	7 Jan. 59		5.5		250	thyroidectomy		150
W. R.	59	M	68	176	1.93	14 Mar. 58	−21	2.7	3	405	Post	13	None
				165	1.87	7 Jan. 59	−10	5.5		207	thyroidectomy		120
A. F.	57	F	62	157	1.71	8 Oct. 57	−16		2	732	Post	40	None
				124	1.55	22 Sept. 58	−13			325	thyroidectomy		90
Cl. P.	35	M	67	138	1.72	4 Dec. 57		3.4	2	260	Post	1/12	None
				139	1.72	18 Feb. 58		7.5			hypophysectomy		120
C. P.	50	M	69	156	1.85	3 Mar. 58	−20	2.1	6	257	Pituitary	2	None
L. M.	66	F	66	199	1.98	11 Sept. 58	−26	2.3	5	220	Primary	10	None
E. D.	60	M	70	186	2.00	4 Mar. 59	−39		4	400	Primary	13	None
Mean (initial) 16 patients				161	1.79		−27†	2.0‡	3.3	399		8.8	
Mean (before treatment) 13 patients				157	1.76		−28§	1.9‖		431‖			
Mean (after treatment) 13 patients				143	1.70		− 8§	5.9‖		249‖			

* Triiodothyronine (micrograms).
† Mean value represents only 13 patients.
‡ Mean value represents only 14 patients.
§ Mean value represents only 8 patients.
‖ Mean value represents only 11 patients.

TABLE II

Lung volumes in patients with myxedema but no lung disease

Patient	Date of study	Inspiratory capacity		Expiratory reserve volume		Vital capacity		Residual volume		Total lung capacity		Residual volume/ total lung capacity ratio ×100
		ml.	%*	*ml.*	%*	*ml.*	%†	*ml.*	%*	*ml.*	%*	
V. C.	20 June 58	2,080	84	1,330	160	3,410	103	1,660	164	5,070	118	33
	15 Sept. 58	1,700	69	1,410	170	3,130	95	2,530	250	5,660	131	45
L. A.	23 June 58	1,520	63	700	88	2,220	69	1,920	79	4,140	74	46
	23 Dec. 58	1,390	58	870	109	2,260	71	1,980	81	4,240	75	47
R. T.	27 June 58	2,670	79	2,940	260	5,570	124	2,600	188	8,170	139	32
	8 Oct. 58	3,430	101	2,390	212	5,820	129	2,090	151	7,910	134	26
M. Mc.	23 Jan. 58	2,280	89	870	102	3,210	94	1,110	46	4,320	74	26
	7 Oct. 58	2,460	96	1,140	134	3,600	106	1,710	70	5,310	91	32
H. H.	18 Sept. 58	2,130	86	2,150	262	4,280	130	1,700	168	5,980	139	28
	19 Feb. 59	2,720	110	1,430	174	4,150	126	1,890	187	6,040	140	31
M. M.	10 Dec. 57	2,280	98	660	86	2,860	92	1,700	70	4,560	82	37
	27 Mar. 58	2,150	92	450	58	2,600	84					
W. P.	16 Dec. 57	2,630	91	230	24	2,770	72	990	102	3,760	78	26
	23 Apr. 58	2,740	94	250	26	2,990	77	1,520	157	4,510	93	34
	1 Oct. 58	2,620	90	880	91	3,500	90	1,220	126	4,710	97	26
	10 Dec. 58	2,530	87	1,180	122	3,710	96	1,610	166	5,320	110	30
M. R.	27 May 58	2,100	88	1,420	178	3,520	110	1,770	73	5,290	94	33
	30 Sept. 58	2,330	97	1,220	153	3,550	111	2,270	93	5,820	103	39
A. C.	14 July 58	2,080	91	640	84	2,720	89	1,410	152	4,130	104	34
	18 Feb. 59	2,250	98	410	54	2,660	87	1,150	124	3,810	96	30
G. D.	17 July 58	2,500	101	1,010	123	3,510	106	2,030	201	5,540	129	37
	7 Jan. 59	2,540	102	820	100	3,360	102	1,790	177	5,150	119	35
W. R.	14 Mar. 58	2,360	73	760	70	3,120	72	3,410	140	6,530	97	52
	7 Jan. 59	3,210	99	860	80	4,070	94	2,730	112	6,800	101	40
A. F.	8 Oct. 57	2,240	95	360	46	2,500	79	1,060	44	3,560	64	30
	22 Sept. 58	2,020	86	480	61	3,110	99	1,540	63	4,650	83	33
Cl. P.	4 Dec. 57	2,450	77	1,190	112	3,570	84	1,760	135	5,330	96	33
	18 Feb. 58	2,750	86	1,260	119	4,010	94	1,340	103	5,350	96	25
C. P.	3 Mar. 58	2,000	61	1,860	171	3,860	88	1,770	132	5,630	98	31
L. M.	11 Sept. 58	2,440	97	250	30	2,690	80	1,560	64	4,250	74	37
E. D.	4 Mar. 59	2,800	84	440	40	3,240	73	1,840	76	5,080	74	36
Mean (initial) 16 patients			85		115		92		115		96	34
Mean (before treatment) 13 patients			86		123		94		124‡		101‡	34‡
Mean (after treatment) 13 patients			91		119		99		131‡		107‡	34‡

* Per cent of predicted normal value. † Per cent of predicted value based on height. ‡ Mean value represents only 12 patients.

TABLE III

Studies of ventilation, respiratory mechanics and diffusion in patients with myxedema but no lung disease

Patient	Date of study	Ventilation: Minute volume (Air) Total	Ventilation: Minute volume (Air) Alveolar	Ventilation: Minute volume (7.5% CO_2) Total	Ventilation: Physiologic dead space		Ventilation: Alveolar gas distribution: 7 min. washout	Ventilation: Alveolar gas distribution: Single breath N_2 test	Mechanical tests: Maximal breathing capacity		Mechanical tests: Maximal expiratory flow rate	Mechanical tests: Maximal inspiratory flow rate	Diffusion	
		L.	*L.*	*L.*	*ml.*	*%**	*% N_2†*	*% N_2‡*	*L./min.*	*%§*	*L./min.*	*L./min.*	*mm. CO/mm. Hg/min.*	*%‖*
V. C.	20 June 58	5.5	3.5	9.1	136	37	0.6	1.5	65	73	214	188	24	96
	15 Sept. 58	8.0	4.9	19.7	194	39	2.2	3.8	73	82	177	162	26	104
L. A.	23 June 58	5.1	2.6	15.9	179	48	2.5	1.3	47	64	136	158	14	58
	23 Dec. 58	6.1	3.2	17.4	159	48	2.6	1.1	55	75	100	171	14	58
R. T.	27 June 58	4.3	2.9	14.6	182	33	1.6	1.1	155	142	361	190	18	58
	8 Oct. 58	5.1	3.5	28.0	200	31	0.8	2.0	156	143	408	291	36	116
M. Mc.	23 Jan. 58	6.4	3.5	12.8	282	45	0.5	2.5	31	42	169	91	10	37
	7 Oct. 58	7.6	5.0	22.7	218	35	0.6	2.7	67	91	200	134	19	70
H. H.	18 Sept. 58	5.8	3.4	16.8	114	33	0.8	1.0	109	122	207	162	27	108
	19 Feb. 59						0.9	0.8	112	125	327	220	38	152
M. M.	10 Dec. 57	5.5	3.0		221	45	4.0	1.5	78	106	240	222	24	109
	27 Mar. 58							1.5	77	105	194	171		
W. P.	16 Dec. 57	6.9	4.3	30.0	314	63	0.5	1.0	49	39	182	70	9	41
	23 Apr. 58	13.0	8.6	40.1	200	34	0.7	0.6	52	41	267	92	10	45
	1 Oct. 58	9.3	4.6	11.7	322	50	1.1	0.5	75	60	182	93	20	91
	10 Dec. 58	10.9	4.7	18.7	248	56	0.5	0.5	100	79	215	151	23	105
M. R.	27 May 58	5.7	3.6	6.4	194	37	0.5	0.5	63	86	240	135	17	71
	30 Sept. 58						0.5	0.6	85	116	325	224	16	67
A. C.	14 July 58	7.1	5.2	24.0	124	44	0.5	2.6	39	44	235	130	19	90
	18 Feb. 59	6.9	4.8	35.9	106	30	0.3	0.8	83	93	270	150	22	105
G. D.	17 July 58	6.3	4.8	18.7	205	24	1.2	4.1	80	90	261	174	20	80
	7 Jan. 59	8.7	7.0	17.5	131	20	1.6	1.2	86	96	248	105	20	80
W. R.	14 Mar. 58	11.7	4.8	16.9	222	35	0.2	2.0	62	68	145	125	18	64
	7 Jan. 59	9.5	5.8	13.8	236	39	0.5	2.3	105	116	290	137	26	93
A. F.	8 Oct. 57	6.3	3.4	21.6	230	46	0.5	3.4	43	59	160	128	11	48
	22 Sept. 58	7.2	4.5	29.8	182	38	0.5	1.0	96	131	214	124	21	91
Cl. P.	4 Dec. 57	8.5	5.3	23.1	294	38	0.0	1.3	86	79	218	207	21	78
	18 Feb. 58	4.9	2.5	12.8	212	49	0.3	3.8	85	78	286	125	22	81
C. P.	3 Mar. 58	12.8	6.2	23.0	264	52	0.8	1.9	105	116	295	225	17	59
L. M.	11 Sept. 58	5.1	3.6	16.2	128	29	0.8	1.2	84	114	174	136	17	65
E. D.	4 Mar. 59	3.8	2.2	17.5	208	43	0.8	1.1	68	75	350	130	11	37
Mean (initial) 16 patients		6.7	3.9	17.8**	206	41	1.0	1.8		82	224	154	17	68
Mean (before treatment) 13 patients		6.8¶	4.0¶	18.7¶	217¶	41¶	0.8††	1.8		78	213	152	17††	69††
Mean (after treatment) 13 patients		7.5¶	4.6¶	21.6¶	189¶	39¶	0.9††	1.7		102	250	167	24††	93††

* Per cent of tidal volume.
† Normal values for seven minute nitrogen washout are less than 2.5 per cent N_2.
‡ Normal values for single breath nitrogen test are less than 1.5 per cent N_2.
§ Per cent of predicted value based on age.
‖ Per cent of predicted value based on height.
¶ Mean value represents only 10 patients.
** Mean value represents only 15 patients.
†† Mean value represents only 12 patients.

TABLE IV

Arterial blood studies in patients with myxedema but no lung disease

Patient	Date of study	O_2 content (rest)	O_2 capacity	O_2 saturation		pCO_2 (rest)	pH (rest)	Hematocrit
				Rest	100% O_2*			
		vol. %	*vol. %*	%	%	*mm. Hg*		%
V. C.	20 June 58	13.30	13.61	97.7	100 + 1.71	38	7.43	33
	15 Sept. 58	15.34	15.88	96.6	100 + 1.99	40	7.37	39
L. A.	23 June 58	13.59	13.04	100 + 0.3	100 + 2.69	44	7.38	36
	23 Dec. 58	13.96	15.27	91.4	100 + 1.20	41	7.40	38
R. T.	27 June 58	10.03	10.31	97.3	100 + 1.94	41	7.42	30
	8 Oct. 58	14.78	14.69	100 + 0.1	100 + 1.86	40	7.41	37
M. Mc.	23 Jan. 58	15.35	15.30	100 + 0.1	100 + 2.21	41	7.33	41
	7 Oct. 58	17.45	18.09	96.5	100 + 1.71	36	7.41	45
H. H.	18 Sept. 58	14.95	15.65	95.5	100 + 1.76	40	7.41	37
	19 Feb. 59							44
M. M.	10 Dec. 57	14.94	15.38	97.1	100 + 3.14	46	7.35	38
W. P.	16 Dec. 57	6.45	6.87	93.9	100 + 1.21	45	7.38	20
	23 Apr. 58	7.39	7.33	100 + 0.1	100 + 1.87	36	7.44	24
	1 Oct. 58	17.64	17.62	100 + 0.02	100 + 1.98	34	7.45	44
	10 Dec. 58	16.46	16.44	100 + 0.02	100 + 2.14	38	7.43	43
M. R.	27 May 58	11.99	12.47	96.2		35	7.50	30
	30 Sept. 58							35
A. C.	14 July 58	16.59	16.19	100 + 0.4	100 + 2.23	32	7.45	40
	18 Feb. 59	16.24	16.06	100 + 0.2	100 + 1.94	33	7.48	40
G. D.	17 July 58	15.83	15.13	100 + 0.7	100 + 2.48	35	7.43	40
	7 Jan. 59	15.70	15.39	98.8	100 + 2.30	36	7.42	43
W. R.	14 Mar. 58	13.76	14.12	97.5	100 + 1.61	36	7.40	33
	7 Jan. 59	17.22	16.39	99.0	100 + 1.98	37	7.41	39
A. F.	8 Oct. 57	13.23	13.65	96.9	100 + 1.58	40	7.38	40
	22 Sept. 58	15.04	14.59	100 + 0.5	100 + 1.87	36	7.39	37
Cl. P.	4 Dec. 57	15.15	15.96	94.9	100 + 1.28	44	7.42	38
	18 Feb. 58	15.75	16.25	96.9	100 + 1.33	37	7.32	38
C. P.	3 Mar. 58	11.02	10.19	100 + 0.8	100 + 2.42	39	7.33	29
L. M.	11 Sept. 58	16.39	17.71	94.1	100 + 1.27	34	7.45	42
E. D.	4 Mar. 59	10.43	11.31	92.2	100 + 1.65	36	7.46	31
Mean (initial) 16 patients				97.1		39	7.41	35
Mean (before treatment) 10 patients				97.8		40	7.40	35†
Mean (after treatment) 10 patients				97.9		37	7.40	40†

* Values following + sign refer to milliliters of O_2 per 100 ml. blood in excess of that required to saturate hemoglobin (*i.e.*, dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.

† Mean value represents 12 patients.

ing capacity was 68 per cent of predicted normal. The maximal expiratory flow rate averaged 135 L. per minute while the mean inspiratory flow rate was 101 L. per minute. The mean D_{LCO} was low, being only 60 per cent of predicted normal. In the four patients studied after therapy, the mean minute volume of ventilation increased from 5.9 to 8.9 L. and the mean alveolar ventilation increased from 2.9 to 5.5 L. The mean maximal breathing capacity increased from 73 to 114 per cent of predicted normal. The mean maximal expiratory flow rate increased from 125 to 275 L. per minute and the mean inspiratory flow rate increased from 90 to 182 L. per minute. The D_{LCO} increased from 14 to 22 ml. CO per mm. Hg per minute (or from 57 to 88 per cent of predicted normal).

The results of arterial blood studies in these patients are shown in the top part of Table VIII. Pulmonary insufficiency for oxygenation and carbon dioxide elimination was present in five of the six patients. The mean arterial oxygen saturation was 84 per cent; the mean pCO_2 was 55 mm. Hg and the mean pH was 7.35. The mean hemato-

TABLE V

Physical characteristics, thyroid function tests, and treatment of: ***A.*** *patients with myxedema and obesity;* ***B.*** *patients with myxedema and lung disease*

Patient	Age	Sex	Ht.	Wt.	Body surface area	Date of study	Basal metabolic rate	Protein-bound iodine	Radio-active iodine uptake (24 hrs.)	Total serum choles-terol	Type of myxedema	Dura-tion of symp-toms	Daily dose of thyroid	Associated disease
			in.	*lbs.*	*M.²*			*μg./100 ml. plasma*	%	*mg./100 ml. serum*		*yrs.*	*mg.*	
A. Patients with myxedema and obesity														
L. R.	67	F	64	388	2.62	14 Mar. 57	− 3	0.7	1	247	Primary	10	None	Obesity
				308	2.34	4 June 57				248			120	
				245	2.16	21 Nov. 57		4.3		287			120	
				223	2.04	7 Apr. 58							120	
				221	2.03	9 Oct. 58	+10	2.8		303			120	
H. K.	53	F	63	277	2.20	22 Jan. 58	−17	2.6	2	432	Primary	20	15	Obesity
				254	2.15	14 Feb. 58		4.7		139			120	
				241	2.10	6 Mar. 58							120	
				239	2.09	15 Apr. 58				305			120	
				250	2.14	3 Sept. 58	+ 4	7.2		283			150	
B. B.	54	M	70	261	2.32	17 Sept. 58	−44	0.5	1	659	Primary	12	None	Obesity
				238	2.23	14 Oct. 58				349			10*	
				231	2.21	4 Nov. 58	−28			272			35*	
				200	2.10	25 Feb. 59	−20			174			50*	
E. K.	66	F	61	240	2.09	12 Sept. 58	+10	2.3	8	344	I^{131}	2	60	Obesity
				219	1.95	21 Jan. 59	+30	3.7		277			120	
K. P.	41	F	65	318	2.43	3 Sept. 57	−21	1.4	2	677	Primary	2	None	Obesity
Mar. R.	67	F	67	279	2.31	25 Sept. 58	−29	2.3	3	407	Primary	5	None	Obesity
Mean (initial) 6 patients				294	2.33		−17	1.6	2.8	461		8½		
Mean (before treatment) 4 patients				292	2.31		−14	1.9†		420				
Mean (after treatment) 4 patients				223	2.06		+ 6	4.6†		259				
B. Patients with myxedema and clinical lung disease														
B. W.	59	F	62	120	1.52	20 Nov. 57	−15	2.9	1	308	Post thyroidectomy	5	None	Pneumonia
M. Mar.	73	F	64	117	1.54	9 Apr. 58	− 1	2.1	3	308	Post thyroidectomy	1 1/12	None	Heart failure
A. L.	54	F	64	167	1.79	24 June 58	−19		2	710	Primary	14	None	Heart failure
W. H.	70	M	71	179	2.00	30 June 58	−41	7.2‡	6.9‡	430	Secondary to iodides	1/3	None	Pulmonary emphysema
Mean 4 patients				146	1.71		−19	2.5§	2†	439		5.2		

* Triiodothyronine (micrograms).
† Mean value represents only three patients.
‡ This patient received iodides prior to this test.
§ Mean value represents only two patients.

TABLE VI

*Lung volumes in: **A.** patients with myxedema and obesity; **B.** patients with myxedema and lung disease*

Patient	Date of study	Inspiratory capacity		Expiratory reserve volume		Vital capacity		Residual volume		Total lung capacity		Residual volume/ total lung capacity ratio ×100
		ml.	%*	*ml.*	%*	*ml.*	%†	*ml.*	%*	*ml.*	%*	
A. Patients with myxedema and obesity												
L. R.	14 Mar. 57	1,240	51	410	51	1,550	48	1,390	57	2,940	52	47
	4 June 57	1,330	55	1,120	138	2,380	73	600	25	2,980	52	20
	21 Nov. 57	1,760	72	1,360	168	2,990	92	940	39	3,930	69	24
	7 Apr. 58	2,080	85	1,040	128	3,220	99	1,530	63	4,750	84	32
	9 Oct. 58	2,430	100	880	109	3,310	102	1,310	54	4,620	81	28
H. K.	22 Jan. 58	1,790	75	860	108	2,570	80	740	30	3,310	59	22
	14 Feb. 58	1,760	73	1,080	135	2,830	88	1,090	45	3,920	70	28
	6 Mar. 58	1,850	77	1,110	139	2,950	92	1,230	51	4,180	74	29
	15 Apr. 58	2,210	92	780	98	2,990	93	890	37	3,880	69	23
	3 Sept. 58	2,200	92	800	100	3,000	94	1,010	42	4,010	71	25
B. B.	17 Sept. 58	2,810	84	920	83	3,730	84	1,950	80	5,680	83	34
	14 Oct. 58	2,850	85	1,100	99	3,950	89	1,530	63	5,480	80	28
	4 Nov. 58	3,280	98	1,170	105	4,450	100	1,830	75	6,280	91	29
	25 Feb. 59	3,530	106	1,820	164	5,350	120	2,220	91	7,570	110	29
E. K.	12 Sept. 58	1,600	69	270	35	1,870	60	1,430	59	3,330	60	43
	21 Jan. 59	1,640	71	390	50	2,030	65	1,640	67	3,670	66	45
K. P.	3 Sept. 57	2,340	94	430	52	2,750	83	910	90	3,660	85	25
Mar. R.	25 Sept. 58	2,120	83	480	56	2,450	72	980	40	3,430	59	29
Mean (initial) 6 patients			76		64		71		59		66	33
Mean (before treatment) 4 patients			70		69		68		57		64	37
Mean (after treatment) 4 patients			92		106		96		63		82	32
B. Patients with myxedema and lung disease												
B. W.	20 Nov. 57	1,390	59	720	91	2,030	64	1,250	51	3,280	59	38
M. Mar.	9 Apr. 58	1,120	46	960	119	2,080	64	2,220	91	4,300	76	52
A. L.	24 June 58	1,240	51	160	20	1,400	43					
W. H.	30 June 58	3,040	90	1,150	102	4,190	93	3,860	159	8,050	116	48

* Per cent of predicted normal value.
† Per cent of predicted value based on height.

crit was 43 per cent. Four patients were studied after treatment. The mean arterial oxygen saturation increased from 80 to 93 per cent; the mean pCO_2 decreased from 61 to 40 mm. Hg and the pH increased from 7.32 to 7.40. The mean hematocrit decreased from 44 to 40 per cent.

C. Patients with myxedema and clinical lung disease

This group consists of four patients with myxedema and clinical lung disease. One woman had pneumonia; two women had pulmonary edema secondary to heart disease and one man had pulmonary emphysema. The results of pulmonary function studies and arterial blood studies in these patients are listed in the lower half of Tables VI, VII and VIII.

DISCUSSION

Lung volumes

In contrast to previous investigators (25, 26), we found the vital capacity was essentially normal in 16 patients with uncomplicated myxedema, regardless of the cause. Our results are similar to those obtained by Schnitker, Van Raalte and Cutler (27). In patients with angina pectoris they induced myxedema by total thyroidectomy. Their patients had a normal vital capacity after induction of myxedema. Our patients with myxedema and obesity had moderate reduction in inspiratory capacity, expiratory reserve volume, vital capacity, residual volume and total lung capacity. Obese patients without myxedema or lung disease have reduced lung volumes (2). We think that when the two diseases coexist the reduced lung volumes are secondary to obesity. When the obese patients lost weight, their lung volumes returned to normal (Table VI).

Ventilation

Patients with myxedema alone ventilated adequately as measured by minute volume of ventilation, arterial pCO_2 and arterial O_2 saturation. They had a lower minute volume of ventilation following the stimulus of breathing 7.5 per cent carbon dioxide in air than did a group of normals. In the normals, the mean minute volume during the third minute of carbon dioxide breathing was 31.2 L. as compared to 17.8 L. for the 16 patients with myxedema alone. Whether this diminished response represents primary depression of the respiratory center in the brain or inability of the chest bellows to respond adequately to the stimulus is unknown. Treatment of the myxedema resulted in a small but not significant increase in the minute volume of ventilation of these patients in response to breathing 7.5 per cent carbon dioxide in air.

Four of the six patients with myxedema and obesity had alveolar hypoventilation manifested by increased arterial pCO_2 and low alveolar ventilation. The existence of alveolar hypoventilation in any patient must be caused by lung disease, malfunction of the chest bellows, inadequate neuromuscular coordination, or a central nervous system lesion. The physiologic problem is to identify which of these four causes the derangement of the function. Lung disease and disease of the bony thorax are excluded in these patients. The trouble must be either in the respiratory center in the brain, the muscles of respiration, or neuromuscular coordination singly or in combination. We have no proof of what comes first or of how many mechanisms are involved. We suspect that the muscles of respiration and neuromuscular coordination are impaired. The evidence which favors this is that patients with myxedema alone do have a significantly reduced maximal breathing capacity. The added burden of obesity results in further reduction in maximal breathing capacity and slowing of both expiratory and inspiratory flow rates. The process is reversible because treatment of the patients with myxedema and obesity resulted in restoration of normal alveolar ventilation in most patients. This took place gradually and over a period of several months as illustrated by L. R., H. K. and B. B. (Tables V through VIII).

Mechanics of breathing

The patients with myxedema alone had reduced maximal breathing capacity which increased significantly following therapy. The ability of a patient to perform this test well is dependent on the cooperation of the patient, the force of the respiratory muscles, and the patency of the respiratory airways. We did not measure airway resistance. We believe the patients were cooperative. We

TABLE VII

*Studies of ventilation, respiratory mechanics, and diffusion in: **A.** patients with myxedema and obesity; **B.** patients with myxedema and lung disease*

Patient	Date of study	Ventilation: Minute volume (Air) Total	Ventilation: Minute volume (Air) Alveolar	Ventilation: Minute volume ($7.5\ CO_2$) Total	Ventilation: Physiologic dead space		Ventilation: Alveolar gas distribution: 7 min. washout	Ventilation: Alveolar gas distribution: Single breath N_2 test	Mechanical tests: Maximal breathing capacity		Mechanical tests: Maximal expiratory flow rate	Mechanical tests: Maximal inspiratory flow rate	Diffusion	
		L.	*L.*	*L.**	*ml.*	%†	% N_2‡	% N_2§	*L./min.*	%‖	*L./min.*	*L./min.*	*ml. CO/mm. Hg/min.*	%¶
A. Patients with myxedema and obesity														
L. R.	14 Mar. 57	8.5	2.6		126	70	1.3	1.8	70	95	46	64	7	29
	4 June 57	12.0	6.2	27.4	240	52	0.5	0.5	94	127	214	103	8	33
	21 Nov. 57	10.6	4.4		295	59	0.4	0.2	111	150	286	125	15	62
	7 Apr. 58	10.4	5.5	20.1	231	47	0.5	1.2	103	139	333	136	15	62
	9 Oct. 58	9.6	5.6	20.3	197	42	0.6	1.4	127	172	365	188	24	99
H. K.	22 Jan. 58	5.1	2.6	13.3	175	49	0.8	3.6	63	85	203	64	16	68
	14 Feb. 58	6.0	3.6		145	40	0.6	1.8	79	107	207	77	21	89
	6 Mar. 58	5.9	3.0		183	52	0.7	1.0	81	109	188	105	19	81
	15 Apr. 58	6.2	3.7	14.6	128	39	0.6	0.6	90	122	156	92	16	68
	3 Sept. 58	9.3	6.0	16.8	171	35	1.8	1.9	86	116	343	140	18	77
B. B.	17 Sept. 58	4.0	1.7	3.3	201	57	6.2	5.4	43	47	132	114	19	64
	14 Oct. 58	4.5	1.9	5.0	200	57	2.0	4.7	50	55	164	184	19	64
	4 Nov. 58	3.8	0.8	5.5	455	76			62	58	184	106		
	25 Feb. 59	6.3	4.2	15.5	257	34	2.6	3.1	70	77	258	177	26	88
E. K.	12 Sept. 58	5.8	3.0	12.3	180	48	1.5	3.8	48	65	120	116	15	69
	21 Jan. 59	5.8	3.5	12.6	130	41	0.9	2.7	55	74	186	80	26	120
K. P.	3 Sept. 57	7.7	3.7	11.7	200	42	1.7	1.5	57	64	207	136	20	79
Mar. R.	25 Sept. 58	7.2	3.5	25.2	192	52	3.1	0.4	38	51	100	113	14	52
Mean (initial) 6 patients		6.4	2.9	13.2**	179	53	2.4	2.8		68	135	101	15	60
Mean (before treatment) 4 patients		5.9	2.5	9.6††	171	56	2.5	3.7		73	125	90	14	58
Mean (after treatment)		7.8	4.8	15.0††	189	38	1.5	2.3		110	288	146	24	96
B. Patients with myxedema and lung disease														
B. W.	20 Nov. 57	4.3	2.4		218	44	1.9	4.6	31	42	51	111	9	40
M. Mar.	9 Apr. 58	3.2	1.3	6.0	136	59	1.0	2.5	28	38	94	75	24	99
A. L.	24 June 58	8.8	4.1	16.9	186	53			25	34	98	42		
W. H.	30 June 58	8.8	5.4	15.6	366	39	1.7	8.5	53	58	78	100	10	33

* Minute volume after breathing 7.5 per cent CO_2 in air for two minutes.
† Per cent of tidal volume.
‡ Normal values for seven minute nitrogen washout are less than 2.5 per cent N_2.
§ Normal values for single breath test are less than 1.5 per cent N_2.
‖ Per cent of predicted value based on age and sex.
¶ Per cent of predicted value based on height.
** Mean value represents only five patients.
†† Mean value represents only three patients.

TABLE VIII

*Arterial blood studies in: **A.** patients with myxedema and obesity; **B.** patients with myxedema and lung disease*

Patient	Date of study	O_2 content (rest)	O_2 capacity	O_2 saturation		pCO_2 (rest)	pH (rest)	Hematocrit
				Rest	100% O_2*			
		vol. %	*vol. %*	%	%	*mm. Hg*		%
A. Patients with myxedema and obesity								
L. R.	14 Mar. 57	12.34	15.01	82.2	100 + 1.70	54	7.31	41
	4 June 57	13.35	14.82	90.1	100 + 1.50	42	7.37	38
	21 Nov. 57	13.96	14.64	95.4		42	7.36	37
	7 Apr. 58	14.39	14.94	96.3	100 + 2.09	36	7.42	37
	9 Oct. 58	14.50	15.28	94.9	100 + 1.34	33	7.42	36
H. K.	22 Jan. 58	14.35	18.03	79.6	100 + 0.62	60	7.35	44
	14 Feb. 58	14.67	17.47	84.0	100 + 0.30	48	7.39	43
	6 Mar. 58	14.79	17.35	85.2	100 + 1.09	51	7.37	42
	15 Apr. 58	16.02	18.15	89.9	100 + 0.94	49	7.36	45
	15 Apr. 58†	17.81	18.19	97.9		45	7.37	45
	3 Sept. 58	15.96	16.86	94.7	100 + 1.39	43	7.40	43
B. B.	18 Sept. 58	11.74	15.89	73.9	98.5	82	7.27	44
	14 Oct. 58	12.82	16.88	75.6	94.4	76	7.29	38
	4 Nov. 58	14.22	16.32	87.1	100 + 1.37	63	7.35	43
	25 Feb. 59	14.68	15.71	93.4	100 + 1.23	38	7.46	39
E. K.	12 Sept. 58	16.44	19.58	84.0	100 + 0.42	46	7.37	47
	21 Jan. 59	15.34	17.61	87.1	100 + 1.30	44	7.36	43
K. P.	3 Sept. 57	12.99	14.33	90.6	100 + 0.75	49	7.34	37
Mar. R.	25 Sept. 58	15.99	17.60	90.9	100 + 1.17	38	7.43	42
Mean (initial) 6 patients				83.5		55	7.35	43
Mean (before treatment) 4 patients				79.9		61	7.32	44
Mean (after treatment) 4 patients				92.5		40	7.41	40
B. Patients with myxedema and lung disease								
B. W.	20 Nov. 57	9.48	9.35	100 + 0.1	100 + 3.15	40	7.38	26
M. Mar.	9 Apr. 58	21.83	22.78	95.8	100 + 1.98	36	7.44	57
A. L.	24 June 58	13.30	13.88	95.8	100 + 0.91	36	7.45	36
W. H.	30 June 58	15.82	16.18	97.8	100 + 1.58	30	7.46	40

* Values following + sign refer to milliliters O_2 per 100 ml. blood in excess of that required to saturate hemoglobin (*i.e.*, dissolved O_2). Normal value for dissolved O_2 is 2.00 ml.
† Arterial studies done with patient in sitting position.

suspect that the change in the maximal breathing capacity is caused by changes in the respiratory muscles. We have no evidence to demonstrate whether this is an abnormality in the contractile mechanism of the muscles as postulated by Lambert and co-workers (10) or in the neural conduction or neuromuscular transmission. Treatment of the patients with myxedema alone and those with myxedema and obesity resulted in a return of the mechanical tests to or toward normal, suggesting that the "muscular" lesion is reversible.

Diffusion

Patients with myxedema and no lung disease had a diminished D_{LCO} which improved significantly after thyroid therapy. The D_{LCO} depends on several factors: *1*) the capillary surface available for diffusion, *2*) the thickness of the alveolar capillary membrane and *3*) the total amount of hemoglobin in pulmonary capillary blood and the reaction rate of hemoglobin with carbon monoxide. All three of these factors may be altered in myxedema. The work of Zondek and associates (13), Lange (14), and Baker and Hamilton (15) suggests that the capillaries might be reduced in number and size, and that there might be alterations in the walls of the capillaries. Circulating plasma and total blood volumes are diminished in myxedema (28–30). Therefore it is possible that pulmonary capillary blood volume is reduced. In our 13 patients with myxedema and no lung disease studied before and after therapy, the mean hematocrit increased significantly from 35 to 40 per cent ($p = < 0.05$). The D_{LCO} increased significantly

TABLE IX

D_{LCO}, hemoglobin and hematocrit in Patient E. D. before and after transfusion with washed red blood cells

Date	D_{LCO}	Hemoglobin	Hematocrit	Washed red blood cells
	ml. CO/mm. Hg/min.	*Gm./100 ml.*	%	
11 Mar. 59	10	9.3	31	500 cc.
12 Mar. 59	10	10.7	33	
13 Mar. 59	11	10.8	35	500 cc.
16 Mar. 59	11	11.9	37	500 cc.
17 Mar. 59	10	12.5	39	
18 Mar. 59	11	12.8	40	
19 Mar. 59	9	12.3	41	500 cc.
20 Mar. 59	9	12.4	45	
21 Mar. 59	9	13.2	43	
23 Mar. 59	11	13.2	43	

from 69 to 93 per cent of predicted normal ($p = < 0.01$). Rankin, McNeill, and Forster (31) found D_{LCO} reduced in patients with anemia. In our patients the improvement in the hematocrit parallels the improvement in D_{LCO}, especially in Patients W. P. and W. R. However, D_{LCO} increased with little change in hematocrit in Patients M. Mc. and A. F. Patient E. D., who had myxedema, anemia and a low D_{LCO}, was studied in an effort to clarify this point. His other pulmonary function tests were essentially normal. While still frankly myxedematous his hematocrit was elevated from 30 to 44 per cent by transfusions of washed red blood cells; at the same time D_{LCO} remained essentially unchanged (Table IX). This suggests that there is significant reduction in D_{LCO} which is on the basis of capillary changes in the lungs. This suggestion is supported by the studies of D_{LCO} in patients with myxedema and obesity. Patients with obesity alone have a normal D_{LCO} (2). The four patients with myxedema and obesity had a low D_{LCO} and a normal hematocrit before thyroid therapy and a normal D_{LCO} and slight fall in hematocrit after therapy. We suspect that myxedema produced changes in the capillaries of the lung which result in a lowered D_{LCO}. We do not know if this is a reduction in the total number of capillaries involved in diffusion, a thickening of the alveolar capillary membrane, or both.

SUMMARY AND CONCLUSIONS

We have studied 26 patients with myxedema before treatment and restudied 21 after treatment with desiccated thyroid or triiodothyronine. Sixteen patients had myxedema but no evidence of lung disease, six patients had myxedema and were obese, four patients had myxedema and lung disease.

The lung volumes were normal in the patients with myxedema only. Obese patients with myxedema had moderate reduction in inspiratory capacity, expiratory reserve volume, vital capacity, residual volume and total lung capacity, probably on the basis of obesity. When the obese patients lost weight their lung volumes returned to normal.

Four of six patients with myxedema and obesity had alveolar hypoventilation. The precise mechanism of alveolar hypoventilation is unknown. Lung disease and disease of the bony thorax were absent. This leaves the possibility of malfunction of the respiratory center in the brain, the muscles of respiration or neuromuscular coordination. We suspect that the muscles of respiration or neuromuscular coordination are involved. The maximal breathing capacity was reduced in patients with myxedema and increased significantly after therapy. This suggests that there is a "muscular" lesion which is reversible.

The diffusing capacity of the lungs for carbon monoxide (D_{LCO}) is reduced in patients with myxedema and increases slowly but significantly after therapy. The best explanation for this is pulmonary capillary involvement, either a thickened alveolar capillary membrane or a reduction in the pulmonary capillary bed, or both.

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CHANGES IN THE PHOSPHATE COMPOUNDS OF THE HUMAN RED BLOOD CELL DURING BLOOD BANK STORAGE *

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The normal human red blood cell, when withdrawn from the circulation, contains a substantial pool of water-soluble organic phosphate compounds, most of which are metabolic intermediates whose concentrations are maintained at constant levels by the glucose metabolism of the cell (1). If blood is stored under blood bank conditions in acid citrate dextrose (ACD) preservative, the organic phosphates of the red cell gradually disappear and inorganic phosphate accumulates. The decline of the organic phosphates coincides with a general deterioration of the cell, for example with a decrease in the glycolytic rate, an inability to concentrate potassium ions and a loss of viability measured by post-transfusion survival (2–4). Several observations have been made previously on the individual phosphate compounds during the storage of human blood in ACD (5–8). In the present study advantage is taken of the high resolution capacity of column chromatography on ion-exchange resins to obtain more complete quantitative information about the nature and changes of the phosphorylated intermediates of red cell metabolism.

METHODS

Units of blood were obtained from routine collections at the San Diego Blood Bank using glass bottles containing Formula B (National Institutes of Health) ACD, and were stored at 4° to 6° C. Seventy-five ml. aliquots were removed from the bottles aseptically at approximately weekly intervals for analysis of the red cells by ion-exchange chromatography. No evidence of bacterial contamination was found in the blood at the end of the storage period.

The aliquot of blood was centrifuged, the buffy coat removed and the packed red cells suspended in normal saline and recentrifuged. The washed cells were extracted with trichloroacetic acid and the acid was removed with ether. The extract was passed through a 1×12 cm. column of Dowex 1-×8 chloride (100 to 200 mesh) which was eluted at 2 ml. per minute with hydrochloric acid and ammonium chloride eluants as indicated in the figures. Fractions of approximately 20 ml. were collected and each was assayed for optical density at 260 mμ and for total phosphorus (9). Spectral measurements at the ultraviolet absorbing peaks indicated that most of the material was adenylate. The anthrone (10), carbazole (11) and orcinol (12) reactions were carried out on all fractions of the 0.01 N HCl and 0.1 N NH_4Cl elution sections to determine the content of sugar mono- and diphosphates. Inorganic phosphate was measured (13) in the 0.01 N HCl fractions. Further details of the procedures used for the isolation and extraction of the red cells, for ion-exchange chromatography and for the identification of the phosphate compounds are given elsewhere (1, 14).

RESULTS AND DISCUSSION

Analyses were carried out simultaneously on three units of blood from unselected donors at the blood bank (two male and one female, ages 21 to 32) with normal blood cell counts. The three samples of blood were assayed at 0, 8, 15, 22, 33, 44 and 62 days of storage. The results did not vary by more than 15 per cent and one was selected for presentation of the data. The 0, 15 and 62 day chromatographic patterns are shown in Figures 1 through 3. Figure 4 summarizes the changes observed in the phosphate compounds studied as a function of time.

The zero time assays revealed several marked differences between the cells collected in ACD in the present study and those red cells collected in heparin anticoagulant (1). Apparently a rapid displacement of the normal equilibrium followed shortly after the blood was mixed with the ACD solution. Actually "zero time" refers to two to three hours of *in vitro* life for the red cell, mostly at reduced temperature, including the period required for the washing and centrifugation of the

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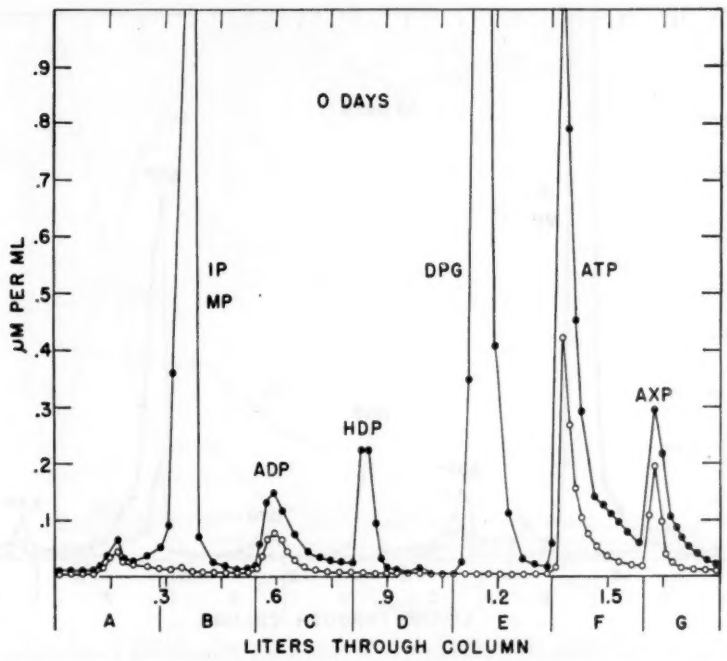

FIG. 1. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS; ZERO DAYS' STORAGE

The red cell extract was passed through a 1 × 12 cm. column of Dowex 1-×8 chloride (100 to 200 mesh) which was eluted at 2 ml. per minute with: **A**, 0.003 N HCl; **B**, 0.01 N HCl; **C**, 0.02 N HCl; **D**, 0.1 N NH_4Cl; **E**, 0.2 N NH_4Cl; **F**, 0.5 N NH_4Cl; **G**, 1.0 N HCl. The data are plotted as μMoles per milliliter of eluate and refer to: total phosphorus, ●; and adenine (from the optical density of 260 mμ, $E = 15 \times 10^3$), ○. The peak values at volumes of 361,378, 1,144, 1,162 and 1,181 ml. were, respectively, 1.1, 1.0, 2.3, 2.9, 2.1 and 1.3 μMoles phosphorus per ml. of eluate.

Abbreviations: IP, inorganic phosphate; MP, monophosphates (difference between IP and total phosphorus in the **A** and **B** elution sections); ADP, adenosine diphosphate; HDP, hexose diphosphate; DPG, 2,3-diphosphoglycerate: ATP, adenosine triphosphate; AXP, unidentified nucleotide.

cells, and so would allow some opportunity for metabolic changes.

The concentrations of both adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (DPG) were slightly lower than normal at zero time and presumably their rapid initial breakdown accounted for most of the extra inorganic phosphate which was about three times higher than normal.

A striking immediate change was the disappearance of fructose diphosphate. It had been found that the hexose diphosphate fraction of the normal human red cell consisted of a mixture of glucose and fructose diphosphates in a ratio of about two to one (1). This was remarkable in that glucose diphosphate does not appear to be present in other tissue in more than trace quantities. That the glucose diphosphate was an active intermediate of glycolysis in the human red cell was demonstrated by its rapid turnover in isotope labeling experiments with carbon[14] and phosphorus[32] (15). In contrast to red cells collected in heparin, the hexose diphosphate of the ACD cells contained no fructose diphosphate at zero time as shown by negative carbazole reaction. The cells, however, had an approximately normal amount of glucose diphosphate, identified by chromatographic position and anthrone and phosphorus ratios.

These relatively rapid changes in the red cell which follow shortly after the addition of blood to

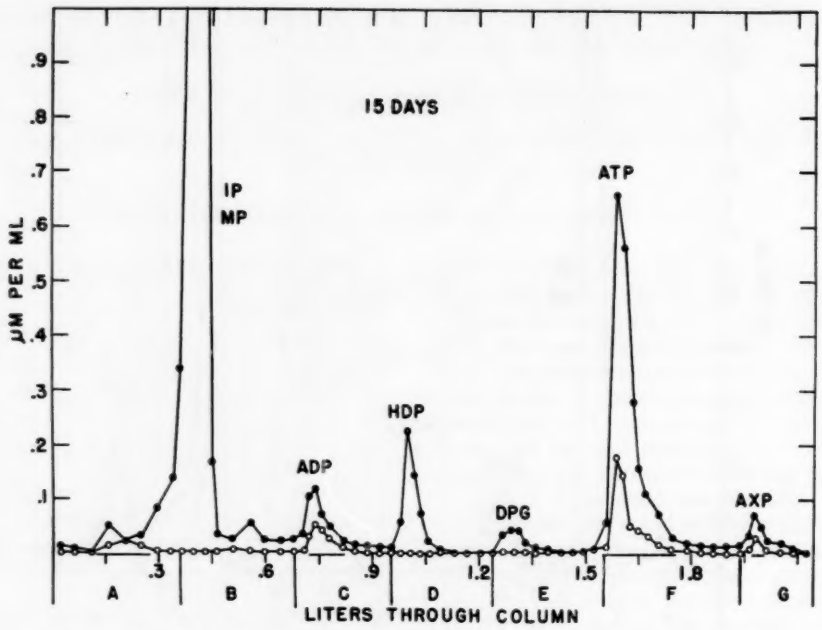

FIG. 2. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS; 15 DAYS' STORAGE

See Figure 1. The peak values at volumes of 388, 410 and 433 ml. were, respectively, 1.1, 2.2 and 2.7 μMoles phosphorus per ml. of eluate.

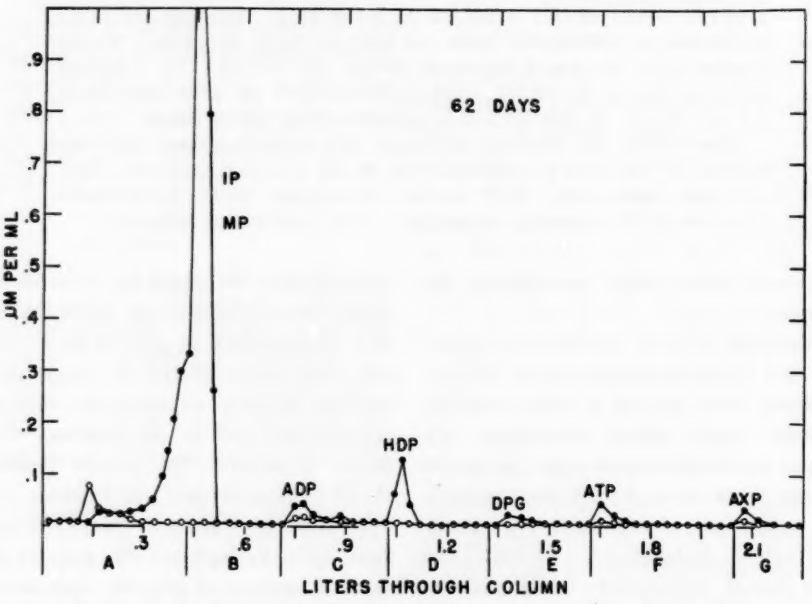

FIG. 3. ION-EXCHANGE SEPARATION OF RED CELL PHOSPHATE COMPOUNDS; 62 DAYS' STORAGE

See Figure 1. The peak values at volumes of 452 and 472 ml. were, respectively, 1.5 and 3.0 μMoles phosphorus per ml. of eluate.

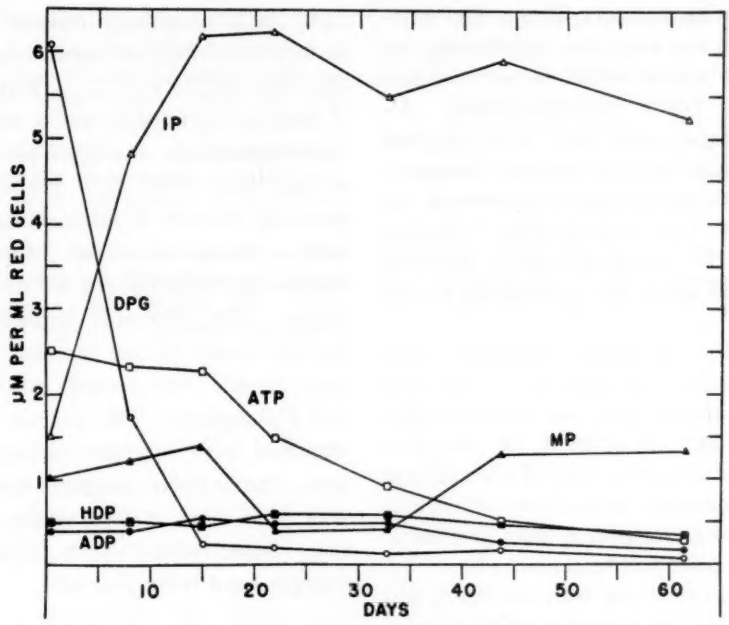

FIG. 4. SUMMARY: CHANGES OF RED CELL PHOSPHATE COMPOUNDS DURING STORAGE IN ACD AT 4° TO 6° C.

The data are taken from ion-exchange chromatographic separations at 0, 8, 15, 22, 33, 44 and 62 days of storage and are plotted as micromoles of phosphorus per milliliter of red cells.

the ACD solution might represent a readjustment of the metabolic equilibrium pattern to the lowered pH rather than reflecting any damage to the metabolic system.

After 62 days of storage, more than 90 per cent of the trichloroacetic acid extractable organic phosphorus had disappeared from the red cell; of special interest was the variable rate of change of the individual compounds.

The precipitous fall in the concentration of DPG during the first 15 days indicated a rapid change of some part of the metabolic machinery. The ATP content decreased slowly during the same period and then fell more rapidly after the DPG had reached a very low level. If the formation of 1,3-diphosphoglycerate were to slow down because of some defect higher in the chain of reactions, then the synthesis of ATP by the transfer of phosphorus from 1,3-diphosphoglycerate to adenosine diphosphate (ADP) would be decreased as would also ATP generation from phosphopyruvate derived from the monophosphoglycerate part of the 1,3-diphosphoglycerate. If monophosphoglycerate is no longer feeding into the phosphoglycericmutase enzyme reaction, a mechanism could be postulated for activating DPG phosphatase to produce 2-monophosphoglycerate and phosphopyruvate and consequent ATP formation, as long as the supply of DPG held out. Assuming a constant rate of controlled metabolic utilization of ATP by the red cell, its net loss would be accelerated when synthesis from DPG stops.

The sudden loss of fructose diphosphate at the beginning of the storage is difficult to evaluate. Maintenance of a stable level of glucose diphosphate throughout most of the storage might result from the absence of an active mechanism for degrading this compound. It should be pointed out that enzymes outside of the main glycolytic pathway must be brought into action to obtain any net synthesis or breakdown of either glucose diphosphate or DPG.

It would be helpful to know more about the concentrations of glucose-6-phosphate and fructose-6-phosphate during the storage period. The organic phosphate in the weakly acidic ester area

(0.01 N HCl elution section) of the chromatograph was variable and relatively high throughout the storage. This section would include the glucose, fructose and ribose monophosphates. Although positive sugar color tests were obtained it is difficult to characterize and quantify these substances without the use of larger aliquots of red cells and formate rechromatography. Further experiments must be performed before anything definite can be said about the components of this fraction.

The intracellular inorganic phosphate rose rapidly to very high levels and at 15 days was probably derived chiefly from the decomposition of the DPG. The concentration of inorganic phosphate inside of the cell leveled off after 15 days even though considerable breakdown of organic phosphate continued, suggesting that the intracellular saturation point had been reached.

The phosphorus-containing material which was eluted with 1.0 N HCl, and labeled AXP (adenine spectrum) in the figures, included, in the zero and eight day analyses, some ATP which had not been completely removed from the column with 0.5 N NH_4Cl. However, there was also present at these times and at the later assays a nucleotide-like substance which has not been identified. Recent studies indicate that a small amount of guanosine triphosphate might be found in this area (16, 17).

It would be premature to interpret the observed changes in terms of any specific metabolic block. Present knowledge of carbohydrate metabolism is very extensive in respect to the nature of the individual enzymatic reactions but is exceedingly limited as to how these reactions are integrated into the delicately controlled metabolic processes within the living cell. Eventually the changes of the metabolic intermediates in such abnormal states as that of blood storage should prove to be a sensitive indicator of the metabolic pathology. However, much more must be learned about what determines the intermediate concentrations in the normal steady state and how they will change under various experimental modifications.

SUMMARY

Phosphorylated metabolic intermediates of the red cell have been assayed by column chromatography on ion-exchange resin at intervals during 62 days of storage of human blood in acid citrate dextrose (ACD) at 4° C. There was a rapid loss of fructose diphosphate and a reduction of adenosine triphosphate and 2,3-diphosphoglycerate on mixing blood with ACD solution with a corresponding increase in intracellular inorganic phosphate. Almost all of the 2,3-diphosphoglycerate disappeared from the red cell during the first two weeks. The adenosine triphosphate concentration fell slowly during the same period and then more rapidly with a much slower loss of adenosine diphosphate. The glucose diphosphate level remained fairly constant throughout the incubation. Intracellular inorganic phosphate rose to a very high level at two weeks with no further rise. Some factors which might influence these changes have been discussed.

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PHOSPHATE COMPOUNDS OF THE RABBIT RED BLOOD CELL DURING STORAGE IN ACID CITRATE DEXTROSE (ACD) AND ACD–INOSINE *

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(Submitted for publication June 8, 1959; accepted September 10, 1959)

Red blood cells of the rabbit appear to react about the same as those of the human to storage in acid citrate dextrose (ACD) preservative in the cold; the cells of both species show a post-transfusion survival of approximately 70 per cent at three weeks and less than 50 per cent at six weeks. The fall in survival is accompanied by a reduction in the glycolytic rate and in the organic phosphate content and these changes are retarded in the presence of certain nucleosides (1–3). These facts suggest that rabbit blood may be substituted for human blood in the study of certain problems related to the maintenance of red cell viability during storage.

The mechanism responsible for the loss of red cell viability during storage is not known, but one reasonable hypothesis would implicate a disturbance in the cell's carbohydrate metabolism. To learn more about the state of the metabolic machinery, which is predominantly glycolytic, rabbit blood was kept in ACD at 4° C. with and without inosine and the phosphorylated metabolic intermediates of the red cells were analyzed by ion-exchange chromatography at the beginning and after three and six weeks of storage.

METHODS

Blood was collected by heart puncture with heparinized syringes from female New Zealand strain rabbits, 2 to 3 Kg. in weight, and was mixed with Formula B (National Institutes of Health) ACD solution. Four mg. of inosine—which had been autoclaved in normal saline—per ml. of blood, was added to half of the pooled blood mixture. One hundred ml. aliquots of the blood mixture were removed for analysis initially and after three and six weeks of storage at 4° C. Aseptic precautions were followed throughout the experimental manipulations and there was no evidence of bacterial contamination at the end of the storage period.

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The aliquot of blood was centrifuged, the plasma and buffy coat withdrawn, and the packed red cells were suspended in two volumes of normal saline and recentrifuged. The washed red cells were weighed, the small amount of saline present being measured by a hematocrit determination, and then were mixed thoroughly with two volumes of 10 per cent trichloroacetic acid and the suspension centrifuged. The residue was extracted with the same volume of 5 per cent trichloroacetic acid. All operations to this point were performed at 0° to 5° C. The acid was removed by extracting with two volumes of ether four times.

The red cell extract was neutralized with 1 N ammonium hydroxide, diluted fourfold and passed through a 1 × 15 cm. column of Dowex 1-×8 chloride (60 to 80 wet mesh) which was eluted at 2.5 ml. per minute with solutions of hydrochloric acid and ammonium chloride, as shown in Figures 1 and 2. Fractions of 20 ml. volume were collected and each was assayed for optical density at 260 mμ and for total phosphorus. The HCl elution fractions were neutralized on the day of collection. The compounds were identified from their elution position in the chromatograph as determined with standards and with the more completely characterized components of chromatographed extracts of fresh rabbit blood drawn into heparin. The identity of the nucleotide peaks was verified by their ultraviolet spectra and by the phosphorus and ribose [orcinol reaction (4)] content. The 0.01 N HCl and 0.1 N NH_4Cl sections of the chromatograph were tested for glucose and fructose mono- and diphosphates by the anthrone (5) and carbazole (6) methods. Sedoheptulose-7-phosphate was identified and quantified by its characteristic reaction in the cysteine-sulfuric acid method (7) as well as by the distinctive colors which it produced in the carbazole, anthrone and orcinol tests. In addition, its elution position was compared with an authentic sample of sedoheptulose-7-phosphate which had been prepared from ribose-5-phosphate with spinach transketolase (8).

For rechromatography, the chloride eluates were diluted fourfold and passed through 1 × 15 cm. columns of Dowex 1-×8 formate (100 to 325 wet mesh) which were eluted at 0.5 to 1.0 ml. per minute with linearly increasing concentrations of formic acid or ammonium formate buffers as indicated in the figures. Further details concerning the procedures which were used for the extraction of the red cells, for ion-exchange chromatography and for the identification of the individual compounds, are given elsewhere (9–12).

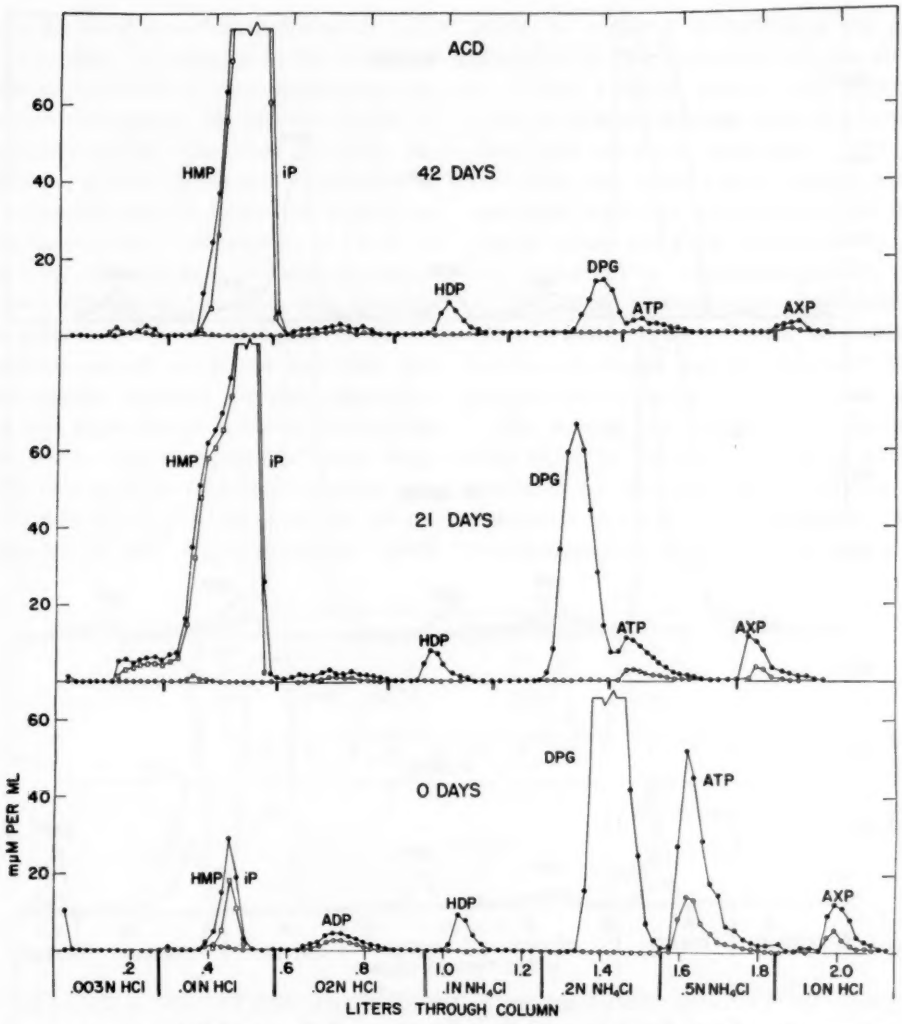

FIG. 1. ION-EXCHANGE CHROMATOGRAPHS OF TRICHLOROACETIC ACID EXTRACTS OF RABBIT RED CELLS STORED IN ACID CITRATE DEXTROSE (ACD) FOR 0, 21 AND 42 DAYS

The data are plotted in millimicromoles per milliliter of eluate (calculated for 1 ml. of red cells) and refer to: total phosphorus, ●; adenine (from the optical density at 260 mμ), ○; and inorganic phosphate, □. The paired vertical lines on the three abscissae indicate where the eluting agent was changed. Abbreviations: HMP, hexose and heptose monophosphate; IP, inorganic phosphate; ADP, adenosine diphosphate; HDP, hexose diphosphate; DPG, 2,3-diphosphoglycerate; ATP, adenosine triphosphate; AXP, unknown nucleotide.

RESULTS AND DISCUSSION

Figures 1 and 2 summarize the results of the ion-exchange analyses of the rabbit red cells which had been stored for zero, three and six weeks in ACD and in ACD plus inosine. The overall picture is one of a marked shift of organic to inorganic phosphate with some significant differences in the pattern in the presence of inosine.

The fall in the level of adenosine triphosphate (ATP) was not associated with any increase in adenosine monophosphate (AMP) or adenosine diphosphate (ADP), probable intermediates.

Since a shift in the ultraviolet absorption maximum was found in the ADP area of some of the chloride chromatographs, these fractions were rerun on Dowex-formate. Figure 3 shows that

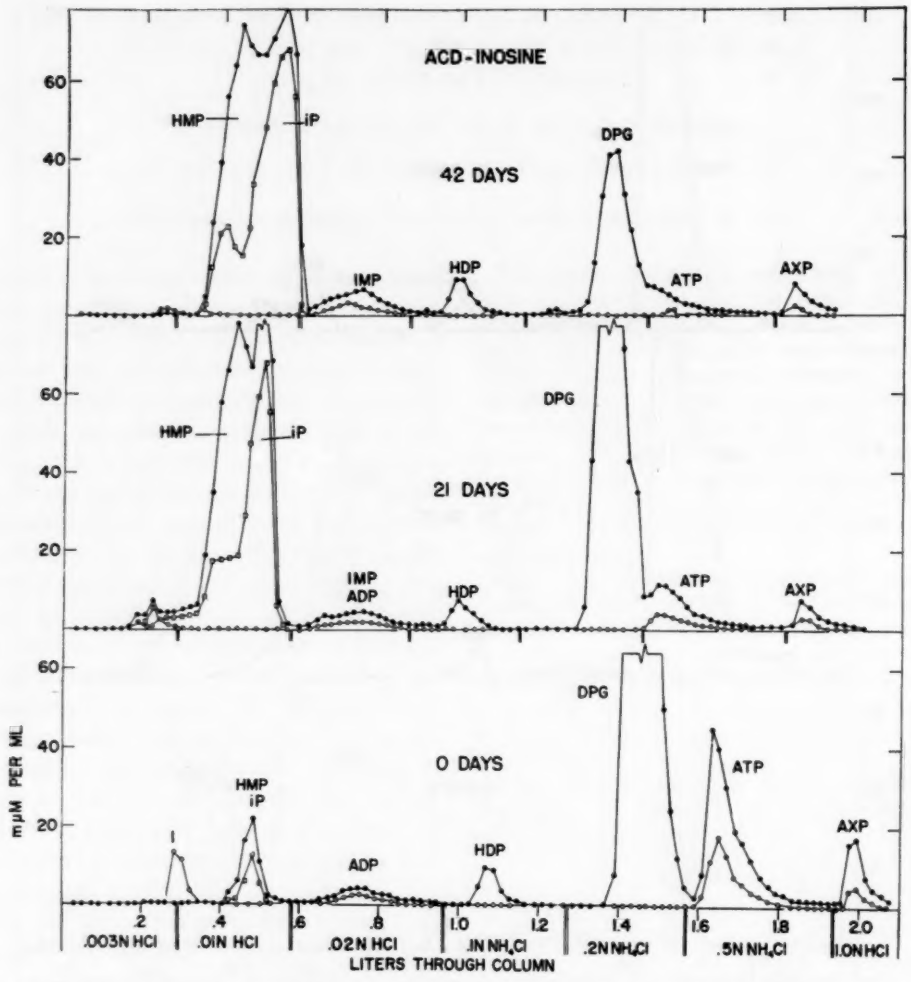

FIG. 2. ION-EXCHANGE CHROMATOGRAPHS OF TRICHLOROACETIC ACID EXTRACTS OF RABBIT RED CELLS STORED IN ACD PLUS INOSINE FOR 0, 21 AND 42 DAYS

See Figure 1. IMP, inosinic acid; I, inosine.

the material consisted predominantly of a mixture of inosinic acid and ADP. It is seen that the loss of ADP was slow in the first three weeks but was accelerated in the second with some sparing effect of inosine. Of interest was the increasing concentration of inosinic acid in the cells stored with inosine. Figure 3 includes a standard chromatograph which shows the elution characteristics of known inosinic mono- and diphosphates and of ADP.

Despite the large quantity of 2,3-diphosphoglycerate (DPG) being decomposed in the blood during storage, there was no evidence for the accumulation of any monophosphoglycerate, phosphopyruvate, pyruvate or glyceric acid, either in the red cells or in the plasma. Presumably the DPG was split to 2-monophosphoglycerate by a phosphatase and then further degraded through phosphopyruvate and pyruvate to lactic acid, the intermediates being metabolized as rapidly as formed.

The hexosediphosphate peak in a chromatograph of normal red cells from blood drawn into heparin includes a mixture of fructose 1,6-diphosphate and glucose 1,6-diphosphate in a ratio of about four to one (13). For some unexplained reason, the fructose diphosphate rapidly disap-

peared from the blood which was collected in ACD or in ACD-inosine. In contrast to the lability of the fructose diphosphate, glucose diphosphate remained stable throughout the storage period in both incubation media. In view of these unusual reactions, it was considered worthwhile to perform additional tests to verify the identity of the glucose diphosphate. An extract of 75 ml. of rabbit red cells, collected in ACD, was chromatographed on a column of Dowex-1 with chloride eluants as described above. The peak in the 0.1 NH_4Cl elution section gave the anthrone and phosphorus results required for glucose diphosphate and was negative for fructose diphosphate (carbazole test). The combined fractions were treated with Dowex-50 to remove ammonium ions and the resulting 0.1 N HCl solution was heated for 10 minutes at 100° C., a procedure which would be expected to hydrolyze the glucose diphosphate at the 1 position without affecting the 6. Figure 4 shows that the acid hydrolysis produced a mixture of equal parts of glucose-6-phosphate and inorganic phosphate. The glucose-6-phosphate was in the correct elution position, had equivalent anthrone (as hexose) and total phosphorus values and gave approximately an equivalent reduction of triphosphopyridine nucleotide (TPN) in the glucose-6-phosphate dehydrogenase assay system. This provides additional support for the conclusion that the substance was in fact glucose 1,6-diphosphate.

The normal red blood cell of the rabbit had been found to contain only a very small concentration of the hexose monophosphates, glucose-6-phosphate and fructose-6- phosphate (13). The hexosemonophosphate elution section of the chro-

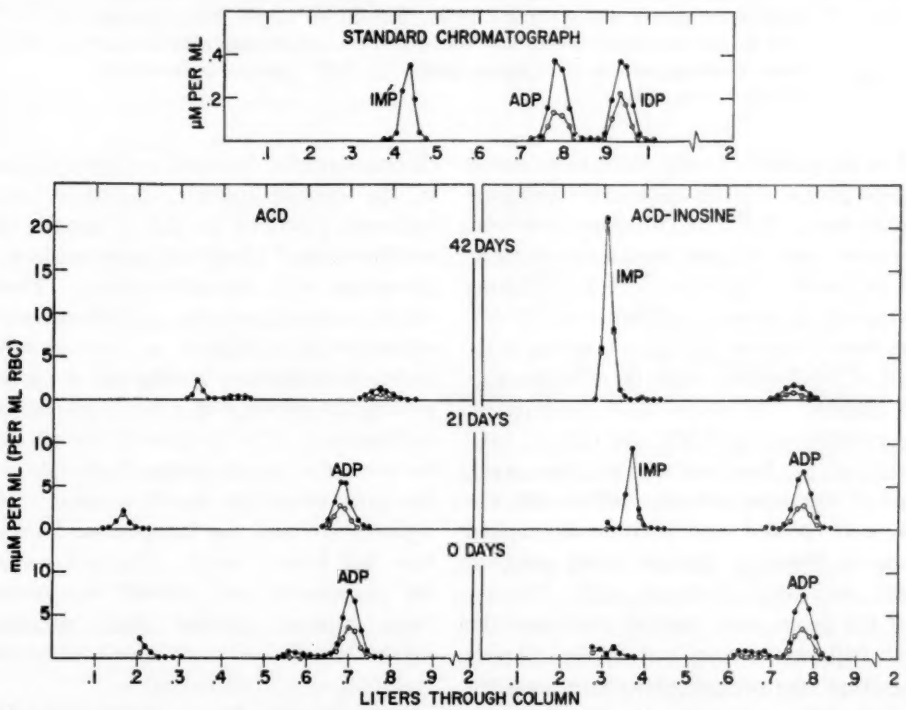

FIG. 3. RECHROMATOGRAPHS OF THE 0.02 N HCL SECTIONS OF THE DOWEX CHLORIDE CHROMATOGRAPHS SHOWN IN FIGURES 1 AND 2

The pooled chloride eluates were passed through 1 × 15 cm. columns of Dowex 1-×8 formate (100 to 325 wet mesh) which were eluted at 1 ml. per minute with 2 L. of linear gradient zero to 2 N, pH 3.6, ammonium formate. Five μMoles of each compound was used in the standard chromatograph. The data are plotted in millimicromoles per milliliter of eluate (calculated for 1 ml. of red cells) and refer to: total phosphorus, ●; and adenine (from the optical density at 260 mμ), ○. For abbreviations, see Figures 1 and 2; IDP, inosine diphosphate.

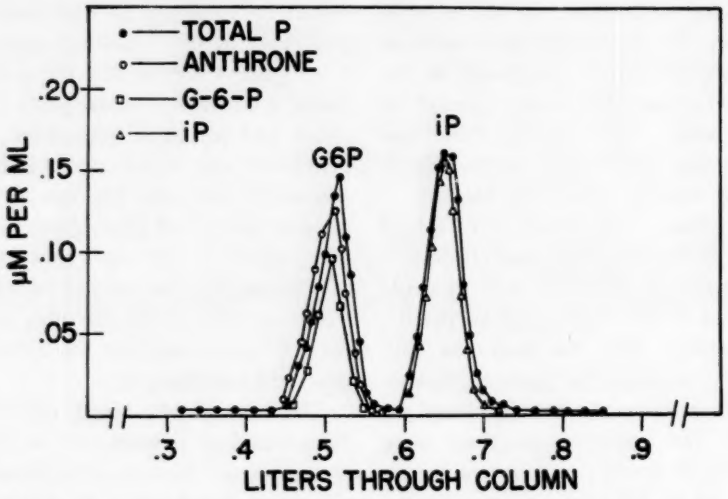

FIG. 4. CHROMATOGRAPH OF ACID HYDROLYSIS PRODUCTS OF GLUCOSE DIPHOSPHATE FROM RABBIT RED CELLS COLLECTED IN ACD

The acid hydrolysis products of the HDP peak of a chloride chromatograph were passed through a 1 × 15 cm. column of Dowex 1-×8 formate (100 to 325 wet mesh) which was eluted at 1 ml. per minute with 2 L. of linear gradient zero to 1 N formic acid. □, G6P (glucose-6-phosphate) dehydrogenase assay.

matograph of the rabbit red cells which were stored in ACD alone gave a weak reaction in the anthrone and carbazole tests. With ACD-inosine, however, an intense color was obtained in the two tests at three and six weeks of storage, with an anthrone reaction atypical of hexoses. Further study disclosed that these fractions also gave a strong reaction typical of ketoheptose with the crysteine-sulfuric acid reagent. To obtain more information about the contents of the 0.003 and 0.01 N HCl elution sections, all fractions of the three week control and of the three and six week inosine experiments were pooled and rechromatographed on columns of Dowex-1 formate using gradient elution with zero to 1 N formic acid. The results from the three week control confirmed the tests which had been carried out in the chloride chromatography: the organic phosphate consisted predominantly of small amounts of glucose-6-phosphate and sedoheptulose-7-phosphate. The three and six week inosine chromatographs were qualitatively the same and the six week data are presented in Figure 5. Almost all of the organic phosphate was eluted as a single peak well in advance of the inorganic phosphate, had the same chromatographic location, and gave the same colors in the cysteine-sulfuric, carbazole, orcinol and anthrone reactions as did a sample of purified sedoheptulose-7-phosphate prepared from ribose-5-phosphate with spinach extract. There was a slight excess of anthrone and phosphorus reacting substance as compared to cysteine-sulfuric and carbazole in the later eluting end of the curve, suggesting the presence of a small amount of glucose-6-phosphate. The section of the chromatograph between the sedoheptulose-7-phosphate and the inorganic phosphate would be occupied by ribose-5-phosphate and the ketopentose-5-phosphates if they had been present. This area was negative for phosphorus and orcinol demonstrating that these expected pentose shunt intermediates of inosine metabolism must have been present in exceedingly small concentrations.

The amounts of some of the phosphate esters which were found at the three storage periods are summarized in Figure 6. The effect of inosine on the maintenance of the organic phosphates of the red cell was not, in some respects, as great as or of a kind that had been anticipated from previous studies (1–3), although most of these had been

performed by incubating the blood at 38° C. with adenosine or inosine at some stage of the storage, and so are not directly comparable. In the present experiments inosine had little influence on the breakdown of ATP. Although there was two or three times as much DPG in the inosine-treated blood at three and six weeks, nevertheless, a major fraction of this compound had been decomposed in both systems. The most striking effect of the inosine was in bringing about a large accumulation of sedoheptulose-7-phosphate at three and six weeks in the apparent absence of ribose, ribulose and xylulose phosphates.

An earlier investigation of the changes of the phosphate compounds of the human red cell during storage in ACD showed a somewhat different pattern of breakdown (14). The DPG had dropped to very low levels at two weeks, at which time there was only a relatively small loss of ATP. The rate of disappearance of the ATP was then accelerated with more prolonged storage. As with the rabbit, the fructose diphosphate of the human red cell disappeared on mixing the blood with ACD while the glucose diphosphate remained elevated throughout most of the storage.

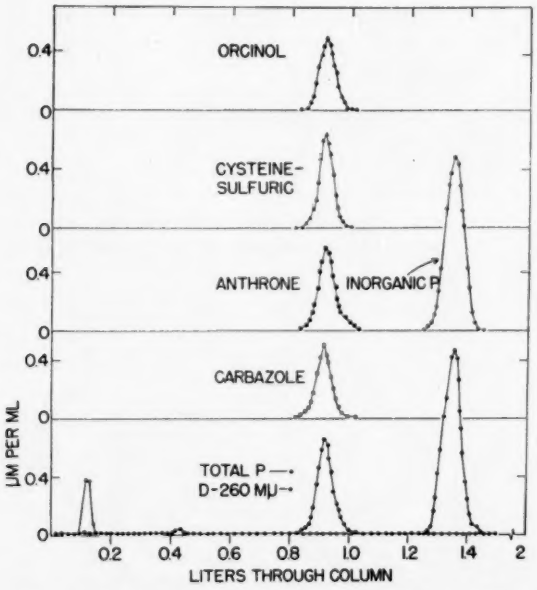

FIG. 5. RECHROMATOGRAPH OF THE 0.003 AND 0.01 N HCl SECTIONS OF THE CHLORIDE CHROMATOGRAPH (FIGURE 2) OF RABBIT RED CELLS STORED SIX WEEKS IN ACD PLUS INOSINE

A 1 × 15 cm. column of Dowex 1-×8 formate (100 to 325 wet mesh) was eluted at 1 ml. per minute with 2 L. of linear gradient zero to 1 N formic acid. First small ultraviolet absorbing peak, inosine; second peak, sedoheptulose-7-phosphate; third peak, inorganic phosphate.

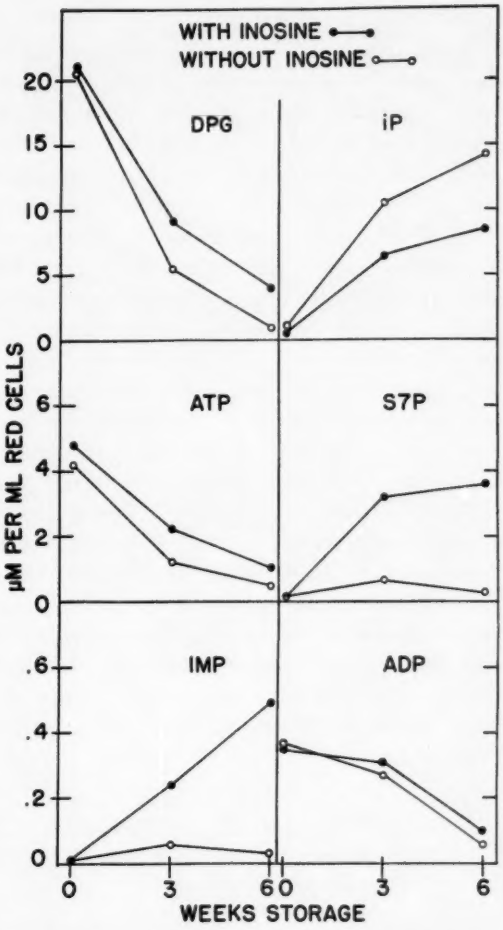

FIG. 6. CHANGES OF PHOSPHORUS COMPOUNDS OF THE RABBIT RED CELL DURING COLD STORAGE IN ACD AND IN ACD PLUS INOSINE

The values are given in micromoles of phosphorus per milliliter of red cells. For abbreviations, see Figures 1 and 2; S7P, sedoheptulose-7-phosphate.

The profound chemical changes taking place in the rabbit red cell during storage in ACD which are recorded here do not yet provide specific answers as to the nature of the metabolic defect, its relationship to red cell viability and the influence of inosine. This new information obtained, however, should prove significant to an eventual solution of these problems.

SUMMARY

The water-soluble phosphate compounds of the rabbit red blood cell have been assayed by ion-exchange chromatography at zero, three and six weeks of storage at 4° C. in acid citrate dextrose (ACD) and ACD-inosine preservative solutions. There was a marked shift of organic to inorganic phosphate during the storage brought about chiefly by the breakdown of 2,3-diphosphoglycerate and adenosine triphosphate, a process which was retarded slightly in the presence of inosine. Adenosine diphosphate showed only a slight drop at three weeks but had almost disappeared at six, again with some slowing of the breakdown given by inosine. Fructose diphosphate disappeared from the red cells immediately upon mixing with ACD, but glucose diphosphate did not change throughout the storage period in either media. There was a relatively large increase in inosinic acid with inosine at three and six weeks. The most striking effect of the inosine was that it produced a large amount of sedoheptulose-7-phosphate at three and six weeks in the absence of a significant accumulation of other pentose shunt intermediates.

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EFFECT OF INOSINE ON THE POST-TRANSFUSION SURVIVAL OF STORED RABBIT ERYTHROCYTES *

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(Submitted for publication June 8, 1959; accepted September 10, 1959)

In 1955 Gabrio, Donohue, Huennekins and Finch (1) reported that inosine prolonged the period that human blood could be stored at 4° C. in acid citrate dextrose (ACD); their experimental evidence indicated greater than 70 per cent viability of the erythrocytes after six weeks of storage. Because of these encouraging findings, a cooperative study was undertaken in which the post-transfusion survival of erythrocytes from blood stored in ACD and in ACD plus inosine (ACDI) was compared (2). Results of this study were not as encouraging as those previously found, and it was reported that only four of the 23 units of blood stored for six weeks in ACDI had better than 70 per cent post-transfusion survival of the erythrocytes. Because of these rather inconsistent results the present study was instituted to learn more about factors which might influence the effect of inosine on the stored erythrocyte. To facilitate a large number and variety of tests, rabbit blood was used since previous work indicated that the results probably would be applicable to human blood.

METHODS

Blood was drawn from one or two female New Zealand rabbits (depending on the quantity of blood to be stored) by intracardiac puncture with heparinized syringes, added to ACD, transferred to siliconized flasks in 5 or 10 ml. volumes, and stored for six weeks at 4° C. Sterile precautions were observed throughout the storage period. Inosine was autoclaved in normal saline unless otherwise stated. In each experiment, ACD blood was stored without inosine as a control and with inosine under various experimental circumstances. At the end of the storage period, post-transfusion survival of the erythrocytes was determined by the double isotope (Cr^{51} and P^{32}) method as described by Gibson and Scheitlin (3). A separate female New Zealand rabbit was used for each post-transfusion survival study.

* Supported in part by the U. S. Army Medical Research and Development Command, Office of the Surgeon General.

RESULTS

Tables I through IV give the 24 hour post-transfusion survival results for erythrocytes of the blood stored six weeks at 4° C. in ACD alone (Table I), and in ACD supplemented with inosine (Tables II through IV).

Fifteen control blood samples stored in ACD alone gave erythrocyte post-transfusion survival values ranging from 4 to 33 per cent with a mean of 18 per cent and a standard deviation of 7.5. Post-transfusion survival of erythrocytes from 49 samples of blood stored in ACDI varied from 28 to 100 per cent with a mean of 61 per cent and a standard deviation of 22. Thus, on the average,

TABLE I

Twenty-four hour post-transfusion survival of erythrocytes stored six weeks at 4° C. in acid citrate dextrose (ACD)

Sample	% 24 Hr. post-transfusion survival	Sample	% 24 Hr. post-transfusion survival
1	9	9	23
2	20	10	25
3	12	11	33
4	12	12	17
5	11	13	23
6	17	14	25
7	20	15	27
8	4	Mean	18
		Standard deviation	±7.5

TABLE II

Effect of inosine on blood stored six weeks at 4° C. in ACD; variation of amount and of time of addition of inosine*

Inosine mg./ml. ACD-blood	Time of addition	% 24 Hr. post-transfusion survival
4.0	0 day	65
4.0	0 day	73
4.0	0 day	93
4.0	21 days	55
4.0	21 days	87
2.0	0 day	72
0.4	Semiweekly	100
0.2	Semiweekly	59

* Schwarz Laboratories, Inc., Lot No. 5504.

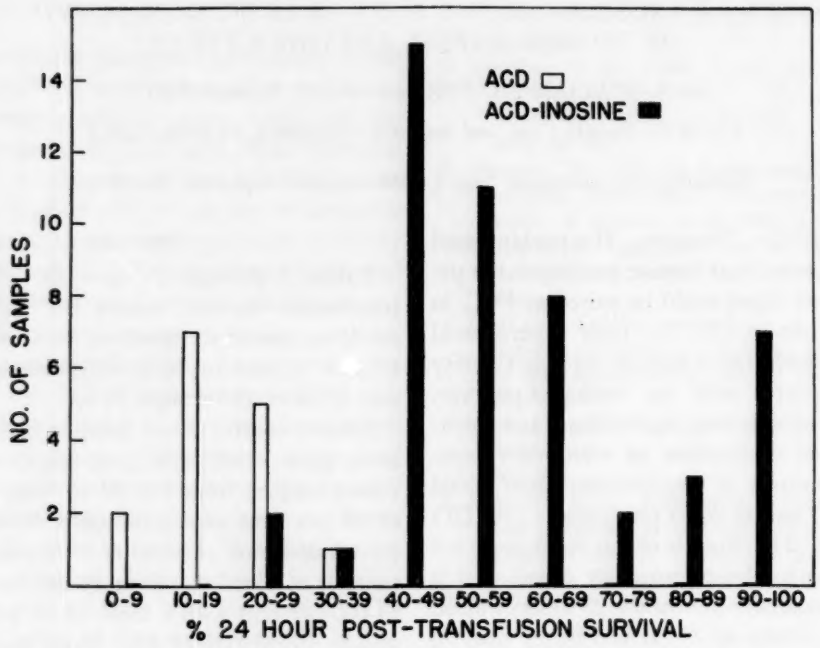

FIG. 1. INCIDENCE GRAPH OF 24 HOUR POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES STORED FOR SIX WEEKS AT 4° C. IN ACID CITRATE DEXTROSE (ACD) AND IN ACD-INOSINE

Mean 24 hour post-transfusion survival of erythrocytes from 15 samples of blood stored in ACD was 18 per cent ± 7.5, and from 49 samples of blood stored in ACD-inosine was 61 per cent ± 22.

3.4 times as many erythrocytes were viable after six weeks of storage when ACD was supplemented with inosine. The results are shown graphically in Figure 1.

The post-transfusion survival of erythrocytes from several samples of stored blood was followed past 24 hours and the curves, presented in Figure 2, show, in agreement with other investigators, that nonviable cells are rapidly removed from the circulation, and that those remaining after 24 hours disappear at approximately a normal rate.

TABLE III

Effect of inosine on blood stored six weeks at 4° C. in ACD; variation in autoclaving and formula of ACD*

Inosine mg./ml. ACD-blood	ACD formula	Inosine solution autoclaved	% 24 Hr. post-transfusion survival
4.0	B	Normal saline	52
4.0	B	Normal saline	69
4.0	B	ACD	52
4.0	B	ACD	66
4.0	A	Normal saline	35
4.0	A	Normal saline	81
4.0	A	ACD	53
4.0	A	ACD	69

* Schwarz Laboratories, Inc., Lot No. 5504.

The following variables were investigated: *1*) addition of inosine at various times during the storage period, *2*) periodic shaking of the blood, *3*) autoclaving inosine in ACD or in normal saline, and *4*) addition of different lots of inosine manufactured by several suppliers. There were no clear-cut differences which would allow the conclusion that any of these factors significantly influenced erythrocyte viability during storage.

Samples of inosine were examined on columns of Dowex-1 formate after autoclaving in ACD or in normal saline. In both cases the compound was eluted quantitatively as a sharp band in the position expected, analyzed correctly by ultraviolet absorption and the orcinol method, and therefore showed no evidence that any breakdown of the nucleoside was produced.

TABLE IV

Effect of inosine (4 mg. per ml. ACD-blood) on blood stored six weeks at 4° C. in ACD; variation of inosine preparation

Inosine preparation	% 24 Hr. post-transfusion survival	Inosine preparation	% 24 Hr. post-transfusion survival
S-5705*	44	S-5504	66
S-5705	49	S-5504	60
S-5601	40	S-5504	65
S-5601	51	S-5706	49
S-5602	40	S-5706	65
S-5602	57	S-5706	48
S-5605	40	S-5706	51
S-5605	46	S-Crude†	41
S-5606	43	S-Crude	84
S-5606	58	S-Crude	95
S-5702	28	S-Crude	97
S-5702	56	P-5706‡	100
S-5703	40	P-5706	92
S-5703	49	P-2303	100
S-5704	40	P-2303	29
S-5504	48	C-100898§	40
		C-100898	52

* Schwarz Laboratories, Inc.
† Schwarz Laboratories, Inc., unpurified inosine preparation.
‡ Pabst Laboratories.
§ California Corporation.

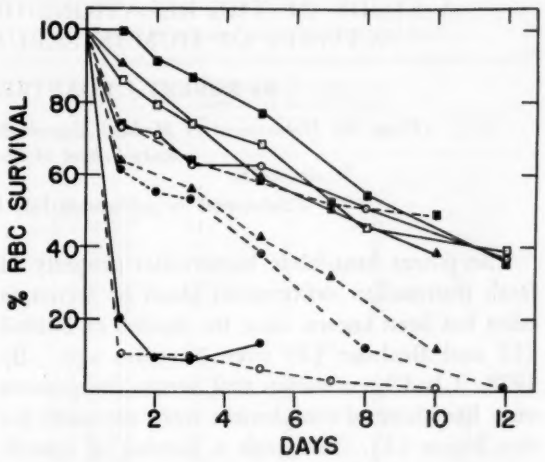

FIG. 2. POST-TRANSFUSION SURVIVAL OF ERYTHROCYTES STORED SIX WEEKS IN ACD AND IN ACD-INOSINE (Cr^{51} AND P^{32} METHOD)

Graphic representation of per cent survival of calculated erythrocytes transfused, ○ - - - - ○ and ●——● —control blood samples stored in ACD. Others, blood samples stored in ACD-inosine.

DISCUSSION

The results are distinguished by a marked variability in the survival of the erythrocytes stored in ACD either with or without inosine. It is not possible to ascertain from these experiments how much of the variation was due to the blood itself and how much to some influence of the experimental manipulations. It will be difficult to assess any of these inconstant effects of inosine or other changes in incubation solution or technic without more information about erythrocyte viability after extended storage periods in ACD alone.

It is obvious, nevertheless, that inosine has a favorable influence on the survival of the rabbit erythrocyte when stored in ACD, and it is felt that further investigation of the effect of various nucleosides on stored blood is warranted.

SUMMARY

Twenty-four hour post-transfusion survival of erythrocytes was determined on 64 samples of blood stored for six weeks at 4° C. in acid citrate dextrose (ACD) or in ACD plus inosine (ACDI). Erythrocyte post-transfusion survival values of 4 to 33 per cent and 28 to 100 per cent for blood stored in ACD and in ACDI, respectively, were obtained.

Inosine has a beneficial effect on the viability of stored blood although there is considerable variability in this effect.

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A STUDY OF THE RELATIONSHIP OF THE NORMAL BACTERICIDAL ACTIVITY OF HUMAN SERUM TO BACTERIAL INFECTION *

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(Submitted for publication July 13, 1959; accepted August 12, 1959)

The potent heat-labile bactericidal property of fresh mammalian defibrinated blood or serum *in vitro* has been known since the studies of Nuttall (1) and Buchner (2) over 70 years ago. By 1928, it had been shown that serum components very like those of complement were necessary for this action (3). Although a number of investigators in the fields of bacteriology and immunology have interested themselves in this bactericidal power of fresh serum, little notice has been taken of it in clinical medicine, probably, as noted elsewhere (4), because it has been believed to be of doubtful effect *in vivo*.

Interest has been revived in the bactericidal activity of serum by the work of Pillemer, Wardlaw and associates (5, 6). They described a normally occurring serum protein of large molecular weight which they named properdin. They attributed the bactericidal property of serum, in part at least, to the presence of properdin, which acted with the four components of complement in the presence of magnesium to kill the bacteria.

The findings of Wardlaw and Pillemer (6) confirmed those of Mackie and Finkelstein (7) that the bacterial strains sensitive to the effect of serum are principally from species of gram-negative bacilli and that the degree of sensitivity is a characteristic of the strain rather than of the genus. However, whereas Wardlaw and Pillemer have described the action of the properdin system as being nonspecific and unrelated to antibody, Mackie and Finkelstein presented data showing that the absorption of a serum in the cold by a sensitive strain resulted in the removal of the killing effect only for that strain, leaving the effect against other strains intact. The question as to whether the complement-dependent bactericidal effect depends wholly upon a system including properdin or one including an antibody-like substance, or whether it may depend partially on both, is not as yet decided.

The belief that the properdin system plays a role in the native resistance of animals to bacterial infection is based upon the finding that an induced decrease in the serum properdin level of laboratory animals is associated with reduced resistance to bacterial challenge, while increased properdin levels correlate with increased resistance (8–10).

What relationship such changes in properdin level *in vivo* bear to the bactericidal effect of serum *in vitro* is unclear. Nearly all the work on properdin levels and resistance to bacterial challenge has utilized the mouse, an animal whose serum is nonbactericidal *in vitro* owing to the lack of the second component of complement (11, 12). Studies using human sera *in vitro* have indicated that properdin levels of 1 to 10 units per ml. of serum show an equal and optimal activity, while increasing the properdin above this range leads paradoxically to a lesser bacterticidal activity (6). Although properdin levels less than 1 unit per ml. have been reported in human disease, it has not been stated whether these levels altered the bactericidal power of the serum (13).

The present study was initiated to determine whether a lessened bactericidal activity of undiluted serum for gram-negative bacilli could be demonstrated using sera from patients with serum protein abnormalities or with diseases commonly associated with bacterial infections. It soon became clear that such a lack of bactericidal effect rarely, if ever, played a role, and that once the sensitivity of a given bacterial strain was determined to any individual serum, it had a very similar sensitivity to the great majority of other human sera, whether from normal or pathological

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† Formerly Bank of America-Giannini Foundation Fellow, and presently recipient of the Lederle Medical Faculty Award.

subjects. Thus all bacterial strains used could be classified as to their degree of serum sensitivity. The problem as to whether strains resistant to the killing effect of serum were more often cultured from the blood of bacteremic patients than might be expected was then investigated.

MATERIALS AND METHODS

Serum. The human blood specimens used in this study were collected aseptically, allowed to stand at room temperature for about an hour, rimmed and centrifuged. The sera were frozen at −20° C. in 6.5 ml. amounts. The whole procedure was completed within two hours of the time of bleeding.

Culture medium. Peptic digest of liver broth was employed as a medium for preparing frozen stock cultures and for the 20 hour cultures used for inoculation. This broth was prepared by mixing 1,000 Gm. ground hog's liver, 1,000 Gm. ground hog's stomach and 100 ml. of concentrated HCl in 10 L. of distilled water and digesting at 50° C. for 18 to 24 hours. This mixture was boiled for five minutes and filtered through cotton. Four Gm. KH_2PO_4, 3 Gm. glucose and 0.1 Gm. *p*-amino benzoic acid (PABA) were added per L. of this filtrate and the pH was adjusted to 7.6 by the addition of 20 per cent NaOH. This 0.3 per cent glucose peptone-rich broth with a slightly alkaline pH includes the main requisites of a medium in which a bacterial strain can develop its maximum resistance against the heat-labile bactericidal effect of normal serum (14, 15).

Bacteria. The bacterial strains used in the study, with the exception of the standard *Shigella dysenteriae,* were isolated from various human sources by the Infectious Disease Laboratory of Stanford University Hospitals. They were taken from single colonies of the original isolation, grown overnight in peptic digest of liver broth, and then this culture was divided into the desired number of samples and frozen at −20° C. The standard *Shigella dysenteriae* was a laboratory strain agglutinating specifically with antiserum to its species. It was selected because previous workers had used this species (6) and because the sera of few patients in the San Francisco area possess agglutinins against it. It was grown for 24 hours in 150 ml. of peptic digest of liver broth. This culture was divided among 125 test tubes and frozen at −20° C. for use as starter culture for each of the tests to be performed with it.

All strains used were of smooth colonial form and were able to remain in uniform suspension in 0.85 per cent saline at 37° C. for a 24 hour period.

The test for bactericidal activity of serum used throughout this study was set up as follows: A 24 hour broth culture of the chosen test organism was diluted in physiologic saline solution by serial 10-fold dilutions to 10^{-6}. Then 0.1 ml. aliquots of each of the six dilutions, 10^{-1} to 10^{-6}, were distributed into three or four 10 × 1 cm. test tubes. This distribution resulted in a pattern of test tubes in the rack similar to the pattern of the results of two such tests recorded in Table I. Each of the freshly thawed test sera (10 minutes in a 19° C. water bath) was distributed in 1 ml. amounts through one row of the serially diluted inocula. In each test, a serum heated to 56° C. for 20 minutes was tested with the 10^{-4} to 10^{-6} dilutions as shown in Table I to serve as a control to indicate whether the killing in the test was indeed due to a heat-labile factor. Pour plate counts were done on duplicate 0.1 ml. amounts of the 10^{-6} dilution. From these counts the number of bacteria in each inoculum could be calculated. The rack of tubes containing the serum and bacteria was shaken well and placed in a 37° C. water bath for two hours. Loop subcultures were made from each tube to a one-sixth section of a standard Petri dish containing nutrient agar. The same 2 mm. diameter loop which delivered approximately 0.002 ml. of serum was used throughout. Special care was taken to insert the loop to the center of the test tube bottom and withdraw it without touching the sides. If the section was completely covered by growth, the result was recorded as 4+; if one quarter was covered, as 1+; and if less than 50 colonies appeared, these were counted.

TABLE I

Results of two typical bactericidal tests

Size of inoculum*	Panel of fresh active sera from three human donors			Heated serum
	J	G	B	
	Klebsiella 32			
1.6×10^7	1+	1+	1+	
1.6×10^6	11	2	0	
1.6×10^5	0	0	0	
1.6×10^4	0	0	0	2+
1.6×10^3	0	0	0	26
1.6×10^2	0	0	0	2
	E. coli 34			
2.8×10^7	4+	4+	4+	
2.8×10^6	1+	12	10	
2.8×10^5	5	2	2	
2.8×10^4	0	0	0	2+
2.8×10^3	0	0	0	28
2.8×10^2	0	0	0	0

* Determined by plate counts on duplicate aliquots of the 10^{-6} dilution.

Only the tube with the greatest inoculum giving a sterile subculture was taken into account when the sensitivity of a particular bacterial strain was determined or the potencies of the various sera were compared. For determining the sensitivity of a strain, the majority result from the three or four sera tested was taken as the final reading. For instance, in Table I it is estimated that the rate of killing of *Klebsiella 32* is 1.6×10^6 organisms per ml. of serum per two hours even though one of the three sera shows a sterile subculture for an inoculum of 1.6×10^6. As will be seen from the discussion of results, agreement among the rates of killing by various sera was good and little difficulty was experienced in deciding upon the

end point. For the purpose of classifying the sensitivity of the bacteria in those few tests in which a majority of sera was not in agreement the test was rerun, and in each case there was a majority agreement on the second run. In each test at least one of the sera included was from one of five healthy donors whose sera had shown good agreement with each other in bactericidal effect in preliminary tests. This normal serum was never at more than a one tube variance with the majority.

Blood culture technique. Blood cultures from patients were obtained by inoculating 10 ml. of freshly drawn blood into 100 ml. of peptic digest of liver broth. The time of transfer of blood from the vein to the broth did not exceed one minute. Tests *in vitro* indicated that inocula of from one to three bacteria of a sensitive strain remained viable after such treatment when amounts of active serum twice that contained in the blood were present.

RESULTS

I. A comparison of the bactericidal rates of various human sera

Sera from 43 patients and 36 healthy adults were tested by the above method. In every test the heated serum control failed to kill the 1,000 to 4,000 bacteria present in the 10^{-5} dilution of the seed culture. Often an inoculum of 100 to 400 bacteria gave a positive subculture after two hours' incubation in the heated serum.

Except for three instances to be described below, all active sera whether from the healthy donors or patients showed a remarkable uniformity in the rate of killing any particular test strain. The clinical states of the 40 patients whose serum showed no defect in bactericidal power are listed in Table II.

TABLE II

*Clinical state of patients from whom test sera were obtained**

Widespread cancer	5	Lupus erythematosus	2
Lymphomatosis and Hodgkin's disease	4	Weber Christian's disease	1
Myelocytic leukemia	3	Hypogammaglobulinemia, adult transitory	1
Lymphocytic leukemia	3	Recurrent bacterial infections	1
Diabetes	4		
Pyelonephritis and bacteremia	3	Idiopathic leukopenia	1
Uremia, terminal	2	Infectious mononucleosis	1
Nephrosis	2	Congestive heart failure	1
Cirrhosis	2	Fever, unknown origin	1
Infectious hepatitis	2	Anorexia nervosa	1

* Cases 1, 2 and 3 are excluded.

Except for Case 1 (described below), every serum showed the ability to kill the standard strain of *Shigella dysenteriae* at a rate of 1×10^7 to 1.5×10^8 organisms per ml. of serum in two hours. Since these data were so uniform, they are not included in the tables.

Forty of the 43 sera from patients and all the sera from healthy donors were tested against at least one other bacterial strain, and most were tested against more.

TABLE III

Degree of internal agreement in tests using panels of sera from healthy donors and in those using sera from healthy donors and patients

	Tests utilizing only normal serum samples	Tests utilizing normal and patient serum samples		
		Total	Normal	Patient
No. of samples tested	326	162	89	73
No. of disagreements	65	36	14	22
Disagreements more bactericidal	29	17	5	12
Disagreements less bactericidal	36	19	9	10
% Disagreement	20	22	17	30
No. of two tube disagreements	6	5	1	4
Two tube disagreements more bactericidal	2	0	0	0
Two tube disagreements less bactericidal	4	5	1	4
% Two tube disagreements	2	3	1	5.5

Table I indicates the type of result observed in the great majority of tests. As may be noted, a one tube disagreement might well be expected as a matter of chance. The degree of internal agreement in tests using panels of sera obtained from healthy donors and that of tests using panels of both patients' and normal sera is shown in Table III. Data from the three cases to be described have not been included.

Of the 488 serum samples tested, 101 or 20.7 per cent showed a disagreement with the majority result. Only 11 or 2.3 per cent of the total were two tube disagreements; none was greater. None of the serum samples showed a consistent

difference from the majority result when tested with different test strains. When retested against the same strain, the difference was not usually reproducible.

Although the results from tests using sera from patients showed a slightly greater percentage of total differences from the majority result than did those for tests in which normal sera were employed, it was not a significant difference. Furthermore, the variances that did occur show an almost equal distribution of those manifesting more or less bactericidal activity. There are too few two tube variations to attach significance to the greater number occurring using patient sera.

In three cases the patients' sera were definitely less bactericidal than normal sera, but only in Case 1 was this deficiency a general one. In Cases 2 and 3, it was a defect specific for the organism isolated from the patient's own blood stream which was resistant to the patient's serum but not to other human sera.

Case 1. E. C., a 62 year old white man, had plasma cell leukemia diagnosed at autopsy. He was uremic because of leukemic involvement of the kidneys. His history was not noteworthy for frequent infections. Serum electrophoresis showed a typical myeloma peak in the γ-globulin region and the albumin: globulin ratio was 2.0: 7.5. The only infection recognized clinically during his month long stay was a pneumococcal lobar pneumonia with bacteremia controlled promptly by penicillin. At death, both lower lobes showed consolidation. Coccal and bacillary forms were noted microscopically.

His serum, drawn before the lobar pneumonia occurred, was tested three times with the standard strain of Shigella and once each against one sensitive Klebsiella and two moderately sensitive strains of Proteus. Against none of these strains was a bactericidal effect demonstrated. This serum was found to be anticomplementary.

Case 2. O. B., a 67 year old white woman, was admitted with a history of low grade fever and a recurring urinary tract infection for four months. Despite antimicrobial therapy, *Escherichia coli* of identical antibiotic sensitivity patterns grew from three blood cultures over a span of 23 days. At laparotomy six weeks after entry, an adrenal tumor with an abscess containing *E. coli* was removed.

E. coli from two of the blood cultures were tested with seven normal human sera and were found to be moderately sensitive, as shown in Figure 1 where the black circle labeled 3 depicts one of the strains. The upper circle represents the sensitivity of this strain as tested against the seven sera from other donors. The lower circle at the tip of the arrow is the level of bactericidal effect of the patient's own serum. This latter result was obtained from three separate tests on serum samples obtained within several days of the positive blood cultures. Thus, serum other than the patient's could kill this strain at a rate of 2×10^8 bacteria per ml. of serum in two hours while her serum could kill only 200, or in reality may have had no bactericidal effect at all since such a low count would not always be detected by the method used. This patient's serum was, however, fully effective against three other strains of *E. coli,* the standard strain of Shigella and one strain of Proteus.

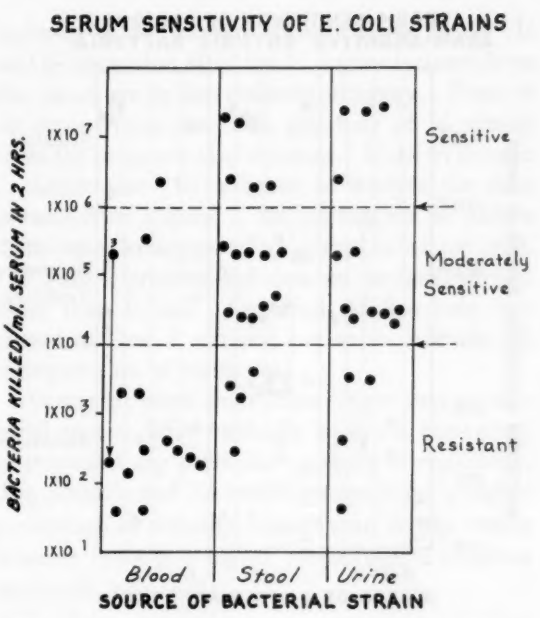

FIG. 1. COMPARISON OF THE DEGREES OF SENSITIVITY TO SERUM BACTERICIDAL EFFECT OF STRAINS OF *E. COLI* ISOLATED FROM THE BLOOD, STOOL AND URINE

Three months later, the patient was readmitted because of recurrent carcinoma and a staphylococcal subdiaphragmatic abscess. At this time her serum, as tested three times, was just as bactericidal for Strain 3 from her previous bacteremia as were the sera from the normal donors.

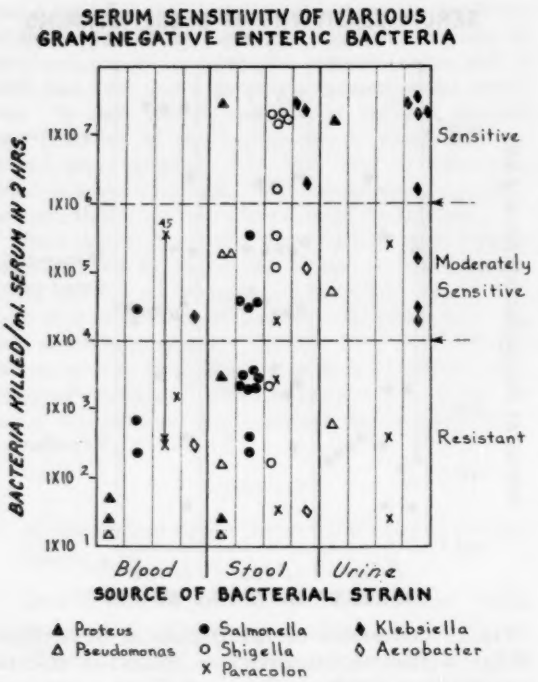

FIG. 2. COMPARISON OF THE DEGREES OF SENSITIVITY TO SERUM BACTERICIDAL EFFECT OF STRAINS OF ENTERIC BACILLI OTHER THAN *E. COLI* ISOLATED FROM THE BLOOD, STOOL AND URINE

Case 3. A. D., a 47 year old white woman, was admitted because of cirrhosis and right pyelonephritis. At entry a strain of "paracolon" bacillus was isolated from the urine and blood. She was treated with tetracycline and chloramphenicol. Her fever subsided after 17 days of treatment. Twenty-two days after entry, an antibiotic-free serum was drawn to test its bactericidal qualities.

The **X** which is labeled 45 in Figure 2 illustrates a situation analogous to that in Case 2. The serum of three normal donors killed at the rate of 4×10^5 and two at a rate of 4×10^6 organisms per ml. of serum in two hours, whereas the patient's own serum was able to kill only 400 organisms, a number barely detectable by this technique. The patient's serum was fully as effective as normal serum against the test strain of Shigella and sensitive strains of Klebsiella and *E. coli.*

In addition to Cases 2 and 3 described above, antibiotic-free sera from five other patients with gram-negative bacillary bacteremia were available for study. Their sera were fully active against sensitive and moderately sensitive test strains.

TABLE IV

Disease states associated with bacteremia from which bacterial strains studied were obtained

	No. of cases		No. of cases
E. coli		Salmonella	
Pyelonephritis	6	Disseminated lupus erythematosus	1
Carcinoma	2	Lymphocytic leukemia	1
Lymphoma	2	Unknown (stool negative)	1
Lymphocytic leukemia	1		
Acute yellow atrophy, liver	1	Paracolon group	
Acute cholecystitis (?)	1	Agranulocytosis	1
Klebsiella		Cirrhosis of liver	1
Lymphoblastic lymphosarcoma	1	Pseudomonas	
Aerobacter		Lymphocytic leukemia	1
Multiple myeloma	1	Proteus	
		Pyelonephritis	2

However, the strains obtained from their blood were resistant to normal serum as well as to their own and thus were not suitable for detecting such a phenomenon as described in Cases 2 and 3.

Thus, in this investigation of differences in the ability of sera from different individuals to kill gram-negative bacteria, only three instances of major differences were found. In one of these it was a lack of killing effect for all test strains and was probably caused by the anticomplementary action of the serum. The other two instances showed a defect specific for the organism with which they were infected and the possible explanation for this phenomenon will be discussed later.

II. A comparison of the sensitivity to serum of bacteria isolated from the blood with those isolated from the stool or urine

Throughout the preliminary stages of the study, it was found that the various test strains evidenced great differences in their sensitivity to the complement-dependent bactericidal effect. Once the sensitivity of a particular strain to one serum was determined, it could be predicted that it would have nearly the same sensitivity to the great majority of other human sera.

It seemed then that a comparison of the sensitivities to serum of bacillary strains isolated from the blood of bacteremic patients to those of strains isolated from the stool or urine might throw some light upon whether the complement-dependent bactericidal effect played any role in native immunity.

Table IV shows the type of disease associated with the bacteremias investigated. The majority of organisms from the stool came from routine cultures and those from the urine were isolated from consecutive positive cultures with no attempt made to distinguish pyelonephritis from cystitis. Neither stool nor urine cultures were from cases with known bacteremia and care was taken to be sure that each specimen came from a different individual.

Figures 1 and 2 contain all the data on the serum sensitivities of the strains of gram-negative bacilli investigated. Three arbitrary zones of sensitivity have been defined, and these form the basis for the categories presented in Table V.

Figure 1 compares the serum sensitivities of strains of *E. coli* isolated from the blood to those of strains from stool and urine cultures. (Strain 3 has been discussed with Case 2 and will not be included in the interpretation of these data.) It will be noted that 10 of the 12 strains isolated from the blood are in the resistant category. Four of 19 strains from the stool, and four of 16 strains from the urine are also resistant. If the Wilcoxon 2 sample test (16) is used to analyze the data presented in Figure 1, the probability of such a distribution's happening by chance is 0.1 per cent.

Figure 2 presents the data on bacterial strains other than *E. coli.* Organism 45 has been discussed in Case 3 and will not be included in the interpretation of these data.

As may be noted from Table V, the various bacterial genera differ markedly as to the percentage of strains in any particular category of sensitivity. The Shigella and Klebsiella groups show a higher percentage of sensitive strains than do the others whereas there is a higher percentage of resistant strains of Salmonella.

TABLE V

Comparison of the degree of sensitivity of bacterial strains isolated from the blood, stool or urine

	Blood	Stool	Urine		Blood	Stool	Urine
E. coli				Aerobacter			
Sensitive	1	5	4	Sensitive	0	0	0
Moderately sensitive	1	10	8	Moderately sensitive	0	1	0
Resistant	10	4	4	Resistant	1	1	0
Total	12	19	16	Total	1	2	0
Salmonella				Proteus			
Sensitive	0	0	0	Sensitive	0	1	1
Moderately sensitive	1	4	0	Moderately sensitive	0	0	0
Resistant	2	8	0	Resistant	2	2	0
Total	3	12	0	Total	2	3	1
Shigella				Pseudomonas			
Sensitive	0	5	0	Sensitive	0	0	0
Moderately sensitive	0	2	0	Moderately sensitive	0	2	1
Resistant	0	2	0	Resistant	1	2	1
Total	0	9	0	Total	1	4	2
Klebsiella				Paracolon group			
Sensitive	0	3	5	Sensitive	0	0	0
Moderately sensitive	1	0	3	Moderately sensitive	0	1	1
Resistant	0	0	0	Resistant	1	2	2
Total	1	3	8	Total	1	3	3

No strain of Shigella from a bacteremia was studied. The percentage of resistant strains of Salmonella isolated from the blood compared to that from the stool was exactly the same. Of the remaining five bacterial groups studied, those strains isolated from the blood showed a higher proportion of resistant strains than those from the stool or urine. Such results agree with the findings from the more extensive study on the strains of *E. coli.*

Considering all the bacteria studied, and excluding the strains of Cases 2 and 3, one finds that there were 21 strains of gram-negative bacilli isolated from the blood. Of these, four were sensitive or moderately sensitive. The two such strains of *E. coli* in Figure 1 came from a case of lymphocytic leukemia and one of diabetes with pyelonephritis. Antibiotic-free serum from neither patient was available for testing. Of the strains of moderate sensitivity depicted in Figure 2, the Salmonella came from a case of lymphocytic leukemia whose serum was not obtained. The strain of Klebsiella was cultured on two different occasions from a case of lymphoblastic sarcoma. This patient's serum was studied and proved to be just as bactericidal for this strain of Klebsiella as was normal serum. His serum was also normally active against two other test organisms.

DISCUSSION

This study was initiated to determine whether major differences in the heat-labile bactericidal effect of individual human sera can be demonstrated and, if such differences exist, whether they can be correlated with an altered susceptibility to bacterial infection. The bactericidal effect was measured by adding bacterial inocula containing varying numbers of bacteria to undiluted serum and noting the surviving organisms after two hours. This method was adopted rather than one using a standard inoculum and varying dilutions of serum because it seemed to simulate more closely the natural conditions under which an infection, and particularly a bacteremia, might occur. Furthermore, since a deficiency of any one of at least six different components will limit the activity of a serum (6, 17), a result from a diluted serum may not reflect the activity of the whole serum. Although the technique employed would not detect minor dissimilarities among sera from various individuals, it seemed evident early that small differences should play little part in affecting native resistance when there were such obvious, large variations among bacterial strains in their sensitivity to the serum.

Except for the three cases described, all sera, whether from normal donors or from patients with diseases in which a serum defect might be suspected to be present, killed any given sensitive bacterial strain at a remarkably uniform rate. All sera were consistently ineffective against resistant strains. Whether the anticomplementary effect of some hyperglobulinemic sera such as that described in Case 1 has any relationship to decreased resistance to infection is unknown. This anticomplementary quality certainly has a profound effect on the bactericidal system *in vitro,* allowing even the most sensitive strains to survive. That the anticomplementary activity of the sera of patients with multiple myeloma interferes with the bactericidal effect *in vitro* has been noted before (6).

In both Cases 2 and 3, sera from patients with prolonged bacteremia lacked bactericidal activity against their own infecting strains but possessed full potency against other test strains. If this finding were caused by a lack of a specific antibody, it would seem that such a deficiency would have appeared occasionally among the sera from the 76 other individuals tested. It seems more likely that this phenomenon is caused by antibody interfering with the bactericidal system (18) and is related to the Neisser-Wechsberg effect (19). The latter describes the inability of a serum containing high concentrations of antibody against a gram-negative bacillus to kill that strain in the presence of complement although the bactericidal effect can be demonstrated if the antiserum is diluted. In the cases described, the sera from both patients were tested at times when antibody in large concentrations should have appeared. The agglutinin titers of their sera were not tested because the possibility of antibody interference had not occurred to us. A situation like that described has been reproduced in the rabbit. As this animal is immunized with a strain of *E. coli,* the undiluted serum loses its normal bactericidal effect for the immunizing strain but retains its activity

for other test organisms (20). What role, if any, this *in vitro* finding plays in native immunity is uncertain.

With the exception of the three cases described above, the sera from individual patients and normal donors were very much alike in their ability to kill any given sensitive test strain. When a strain was resistant to one serum, it was resistant to all the sera tested. In all, antibiotic-free sera from six cases of bacteremia other than Cases 2 and 3 were available for study. All were effective against sensitive and moderately sensitive test strains. In five cases, a lack of effect against the infecting strains, even if present, could not be demonstrated because these strains were resistant to serum. In the other case, the patient's serum was fully active against the moderately sensitive strain of Klebsiella causing the infection as well as against other test strains. Such an occurrence might have several explanations. Perhaps the sensitive organisms were being constantly fed into the blood stream from a protected infected focus. They may have been sheltered by an intracellular location within phagocytes. The bactericidal system may not be nearly as effective *in vivo* as *in vitro*.

On the basis of the evidence presented, it would appear that changes in the bactericidal potency of undiluted serum as measured *in vitro* rarely take place even in the serum of patients with bacteremia or with diseases often associated with infection.

On the other hand, the finding that the great majority of the enteric bacillary strains causing bacteremia were resistant to killing by serum indicates that the system may be of importance as a factor limiting the number of strains capable of invading or persisting in the blood stream. Since the enteric strains causing bacteremia ordinarily gain entrance from either the intestine or urinary tract, the data showing that the majority of strains isolated from these sources are moderately or highly sensitive suggest that there is a limitation of the number of strains capable of causing bacteremia. The finding of one instance in which bacteremia of at least two days' duration was caused by a strain moderately sensitive to the patient's own serum indicates that the limitation is not absolute.

The percentages of resistant and of sensitive strains isolated from the urine were not significantly different from those isolated from the stool. This finding supports the belief that there is actually a difference between strains isolated from these two sources and those from the blood, and is compatible with the belief that the kidney is not ordinarily infected via the blood stream.

From the results of this study, one might hypothesize that one characteristic of a systemically pathogenic enteric bacillary strain would be its resistance to killing by serum. There are few studies to confirm this theory, but those which have been reported are in agreement with it. Jensen was able to derive a strain of *Salmonella typhimurium* nonpathogenic for the mouse from a pathogenic strain by passing it *in vitro* at gradually increasing temperatures of incubation for 206 passages. This strain had the same antigenic qualities and fermentative characteristics as the parent strain (21). Maaløe showed the parent strain to be resistant to, and the derived strain sensitive to, the complement-dependent serum factor (14). He showed the two strains to be equally toxic and infective for the mouse, but the apathogenic variant appeared unable to multiply and spread. Rowley compared the pathogenicity for mice of strains of *E. coli* sensitive to active guinea pig serum and those resistant. He found that the resistant strains were in each instance the more pathogenic (22). In a recent study it has been found that 18 of 19 strains of gram-negative bacilli isolated from burns were resistant to the bactericidal action of normal, active serum (23).

Obviously, the property of resistance to the bactericidal effect of serum does not alone impart pathogenicity to a bacterial strain. However, once given the opportunity of access to the blood stream, the survival of any particular bacterium and its ability to spread may well depend upon such a quality.

Although, as noted above, a more sensitive strain may be derived from a resistant one by artificial methods and sensitivity can vary with the culture medium used to prepare the inoculum (14, 15), we were impressed by the stability of the quality of sensitivity to serum as a characteristic of a strain if the same medium and cultural conditions were

employed. Our attempts to derive a strain significantly more resistant to killing by serum by serial passage through active human serum have thus far met with no success.

It may be noted from Table V and Figure 2 that a high proportion of the strains of Shigella are sensitive, whereas a high proportion of strains of Salmonella are resistant. If, as our results indicate, resistance to serum is of importance to a strain's ability to cause bacteremia, this distribution may be related to the frequency of cases of bacteremia cause by strains of Salmonella and the rarity of such cases caused by Shigella.

SUMMARY

An investigation of the heat-labile bactericidal properties of undiluted sera from 36 healthy donors and 43 patients with diseases often associated with infection has been conducted. With the exception of the sera from three patients, no major difference in bactericidal ability was found among the various individual sera. Two of these three sera showed a specific inability to kill the organism which was cultured from the patient's own blood. It is hypothesized that the inability of the patient's own serum to kill the infecting bacterial strain is due to interference by antibody with the normal bactericidal effect. The third serum was ineffective against any test strain of bacteria employed because it was anticomplementary.

In studying the sensitivities of strains of gram-negative enteric bacilli to the heat-labile bactericidal quality of blood, it was found that a much higher proportion of bacterial strains isolated from the blood, than of those isolated from the stool or urine, was resistant to killing by serum. The hypothesis is presented that the main role of the heat-labile bactericidal system in native resistance to infection is to prevent many strains from invading and persisting in the blood, and that changes in this bactericidal property are infrequent and of less importance.

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THE SURVIVAL OF STRAINS OF ENTERIC BACILLI IN THE BLOOD STREAM AS RELATED TO THEIR SENSITIVITY TO THE BACTERICIDAL EFFECT OF SERUM

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Previous work has indicated that enteric bacillary strains vary greatly in their susceptibilities to the bactericidal effect of active human serum *in vitro* (1–3). Although minor differences in the bactericidal rates of sera from different individuals undoubtedly occur, these differences seem of small import when contrasted to the great variability in the sensitivities of the different strains to such sera (3). If the bactericidal activity of serum as measured *in vitro* reflects a corresponding activity *in vivo*, bacterial strains resistant to it should be much more capable of causing a sustained bacteremia after introduction into the circulation than are sensitive strains.

One survey has indicated that bacteria isolated from the blood of bacteremic patients are much more likely to be relatively resistant to the bactericidal action of serum than are those isolated from the stool or urine (3). Yet strains sensitive to the patient's own serum (3) or defibrinated blood (4) have been isolated from bacteremic patients.

One method of assessing the importance of the complement-dependent bactericidal system of serum relative to other native defense mechanisms might employ the simultaneous intravenous injection of a serum-sensitive and a serum-resistant strain into a laboratory animal. The survival rates of the two strains could then be determined by taking serial blood cultures. This paper will report such experiments.

The rabbit was chosen as a convenient experimental animal although our tests *in vitro* have shown its serum to be somewhat less bactericidal than that of the sheep or the guinea pig. Results to be reported below indicate that rabbit serum is comparable to, although less active than, human serum in its bactericidal activity.

The experiments to be described utilized pairs of strains of gram-negative bacilli. One strain of each pair was sensitive to serum and the other resistant. They could be distinguished from each other by their differing antibiotic sensitivities. Five combinations of eight strains were used, and every pair was tested for sensitivity to human and to rabbit sera *in vitro*. Comparable numbers of each strain of a pair were suspended together in saline solution and injected intravenously into rabbits. The number of viable organisms of each strain remaining in the blood stream at various times after injection was then determined by quantitative blood cultures.

MATERIALS AND METHODS

Tests of human and rabbit sera in vitro. Preliminary experiments to determine the comparability of the bactericidal action of human and of rabbit sera *in vitro* were conducted. The method used was that of subjecting 10-fold dilutions of a 20 hour broth culture of a test organism to the action of 1 ml. of undiluted active serum for a two hour period. The number of organisms killed during this time was determined by loop subcultures to agar (3). The numbers killed by human serum were determined by taking the majority result of a test which included at least three human sera. Ordinarily the action of one rabbit serum was compared to that of the three human sera, but at times, as many as three rabbit sera were included in the test.

Experiments with combinations of bacterial strains. Six strains of *Escherichia coli*, one strain of Klebsiella, and one strain of Aerobacter were used in these experiments. These strains had been isolated from human urine at Stanford University Hospital. Each had been subcultured once in broth for 20 hours; this culture was divided into a number of portions to be used as starter culture, and these were stored at $-20°$ C.

These particular strains were selected from a group known to be sensitive to streptomycin and resistant to tetracycline or vice versa. Strains which had opposite sensitivities to the antibiotics and to killing by serum were then used in the experiments as pairs.

Young adult male rabbits weighing from 2.7 to 3.5 Kg. were used in all experiments.

Rabbit blood was drawn aseptically by cardiac punc-

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TABLE I

Bactericidal action of normal human and rabbit sera in vitro against the bacterial strains used for the injection of the rabbits

Combination	Serum used	Bacterial strains	Percentage of inoculum surviving				
			10 min.	*20 min.*	*30 min.*	*40 min.*	*60 min.*
II	Rabbit	*E. coli* 119R	100	102	100	103	100
		129S	0	0	0	0	0
	Human	*E. coli* 119R	84	67	61	50	33
		129S	0	0	0	0	0
III	Rabbit	*E. coli* 118R	73	56	64	67	131
		192S	1	0	1	1	0
	Human	*E. coli* 118R	64	34	21	24	30
		129S	0	0	0	0	0
IV	Rabbit	*E. coli* 108R	100	100	106	129	165
		130S	32	17	10	5	3
	Human	*E. coli* 108R	100	101	93	108	113
		130S	1	0	0	0	0
V	Rabbit	Aerobacter 125R	96	83	95	90	111
		Klebsiella 27S	5	0	0	0	0
	Human	Aerobacter 125R	92	99	93	92	111
		Klebsiella 27S	2	0	0	0	0

ture, allowed to clot for one hour, and then centrifuged for 20 minutes at 2,000 rpm. The serum was harvested and apportioned in appropriate amounts into sterile serological tubes. These were placed at −20° C. within two hours of drawing the blood.

Human blood was drawn from the antecubital vein of healthy donors and processed in a manner similar to that used for the rabbit blood.

Streptomycin [1] and tetracycline [2] from commercial sources were used to make up the antibiotic plates.

Tests of serum and bacterial combinations in vitro. Each pair of bacterial strains was selected so that one member was serum-sensitive and the other serum-resistant. One was streptomycin-sensitive, tetracycline-resistant, and the other had the opposite antibiotic sensitivities. Each strain was cultured separately by adding a 0.1 ml. portion of the thawed starter culture to a tube containing 4.5 ml. of peptic digest of liver broth and incubating this at 37° C. for 20 hours. The cultures were diluted separately in saline to previously determined suitable dilutions and then combined in volumes which would yield approximately equal numbers of each strain. To a 0.4 ml. aliquot of this combination, 3.6 ml. of active serum was added. This addition resulted in inocula of from 1,000 to 3,000 organisms of each strain per ml. of serum. The mixture was rapidly pipetted up and down 10 times and placed in the 37° water bath. Precisely the same procedure was followed with heated serum in a control tube. At the intervals noted in Figure 1 and Table I, 0.1 ml. aliquots were inoculated into duplicate pour plates containing nutrient agar alone and into plates with agar containing 10 μg. per ml. of tetracycline or streptomycin. Plate counts were done after incubation for 24 hours at 37° C. Since good agreement was found between duplicate plates, the counts were averaged and

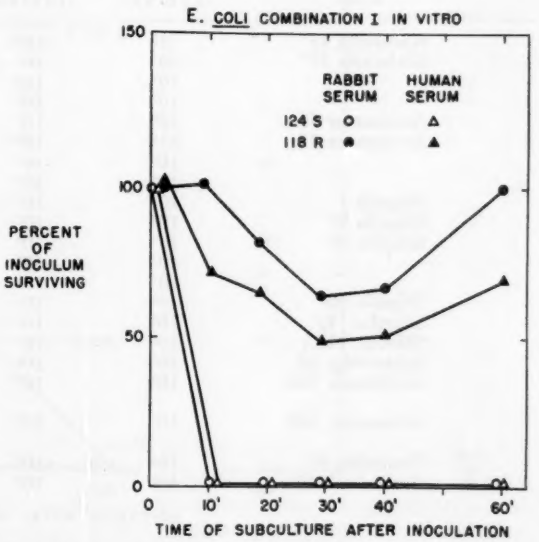

FIG. 1. BACTERICIDAL ACTION OF NORMAL HUMAN AND RABBIT SERA *IN VITRO* AGAINST THE TWO STRAINS OF *E. COLI* MAKING UP COMBINATION I

[1] Streptomycin sulfate, E. R. Squibb & Sons, New York, N. Y.

[2] Achromycin Hydrochloride®, Lederle Laboratories, New York, N. Y.

expressed as the percentage of original inoculum surviving.

For each individual strain, plate counts were performed on plain agar and each of the antibiotic agars to insure that the properties of the strains remained constant. There was no evidence of resistant colonies developing from an antibiotic-sensitive strain, nor of suppression of the streptomycin-resistant strain by streptomycin. However, tetracycline regularly suppressed 10 to 20 per cent of the colonies of strains resistant to it.

Tests of bacterial combinations in vivo. As in the experiments *in vitro*, 20 hour broth cultures were used. These were centrifuged, the supernatant broths removed and the bacteria resuspended uniformly in normal saline solution. A 0.5 ml. aliquot of each suspension was removed for dilution to 10^{-6} for viable bacterial counts. The two strains were then mixed in that proportion which previous experience indicated would yield approximately equal numbers of each for injection into the rabbit.

Injection was made via the marginal ear vein. Blood samples of 0.6 ml. were withdrawn from the veins of the opposite ear at 1, 15 and 30 minutes, and at two hours following injection, using a 1.5 ml. syringe which had been rinsed in heparin. The samples were immediately diluted in sodium citrate-saline solution, the first 1:10, and all others 1:2. Aliquots of 0.1 ml. were removed and plated on nutrient, streptomycin and tetracycline agars. At 24, 48, and sometimes at 96 hours, 8 ml. blood samples were taken by cardiac puncture, diluted 1:2 in sodium citrate, and 1 ml. aliquots plated on nutrient and antibiotic agar.

Representative colonies from each plate showing growth were subcultured in broth and then streaked on each type of antibiotic agar to insure that neither strain had changed in its sensitivity to antibiotics and that the potencies of the antibiotic plates were correct. Several isolates from each type of agar on which growth appeared, following inoculation with blood drawn 24 and 48 hours after infection of a rabbit, were tested for their sensitivities to rabbit and to human serum to insure that the antibiotic plates were distinguishing the strains and to determine whether any change in sensitivity to serum had occurred.

RESULTS

Comparative effects of human and rabbit sera upon gram-negative bacilli in vitro

Table II presents the results from experiments *in vitro* comparing the action of human and of rabbit sera. In all, 46 tests were done on 30 different bacillary strains using sera from 16 different rabbits. Those strains tested more than once were

TABLE II

Comparison of bactericidal effect of normal human and rabbit sera

Bacterial test strain	No. bacteria killed in 2 hours by Human serum (1.5 to 4×)	No. bacteria killed in 2 hours by Rabbit serum (1.5 to 4×)	Bacterial test strain	No. bacteria killed in 2 hours by Human serum (1.5 to 4×)	No. bacteria killed in 2 hours by Rabbit serum (1.5 to 4×)
Klebsiella 15*	10^7	10^7	*E. coli* 34	10^4	10^3
Klebsiella 37*	10^7	10^7	*E. coli* 57	10^5	10^5
	10^7	10^7		10^5	10^4
	10^7	10^7		10^5	10^4
Aerobacter 56	10^5	10^3	*E. coli* 59	10^4	10^1
Aerobacter 75	10^6	10^5	*E. coli* 63	10^4	10^2
	10^6	10^4	*E. coli* 64	10^6	10^3
	10^6	10^4	*E. coli* 72	10^5	10^4
Shigella 1	10^8	10^7		10^5	10^4
Shigella 97	10^7	10^6	*E. coli* 92	10^1	10^1
Shigella 98	10^7	10^6		10^1	10^1
	10^7	10^5	*E. coli* 102	10^3	10^3
	10^7	10^5	*E. coli* 108	10^2	10^1
Shigella 107	10^2	10^1	*E. coli* 119	10^4	10^1
Shigella 142	10^5	10^3	*E. coli* 129	10^7	10^5
Shigella 142A	10^1	10^1		10^7	10^6
Salmonella 20	10^4	10^3		10^7	10^6
Salmonella 106	10^2	10^3	*E. coli* 130	10^6	10^3
				10^6	10^4
Salmonella 149	10^7	10^6		10^6	10^4
				10^6	10^4
Paracolon 95	10^5	10^2		10^6	10^4
Paracolon 100	10^3	10^3		10^6	10^4
			E. coli 160	10^5	10^3
			E. coli 185	10^5	10^3
			E. coli 197	10^2	10^2

* In these tests neither human nor rabbit serum was tested with a greater inoculum so that differences between them would not be evident.

tested against sera from different rabbits. It may be noted that those six strains which were relatively resistant to human serum, being killed at a rate of less than 1×10^4 (designated 10^3 or less in the table) bacteria per ml. in the two hours, were also resistant to rabbit serum. The other strains of moderate or great sensitivity to human serum were usually sensitive, but less so, to rabbit serum. In no case was a strain more sensitive to rabbit serum. As with human sera, little difference was noted in the action of sera from different rabbits. It may be stated in general that rabbit serum is bactericidal for the same bacterial strains as is human serum, though to a lesser degree.

It having been determined that the rabbit was a suitable animal for such experiments, four combinations of six strains of *E. coli* and one combination of strains of Klebsiella and Aerobacter were exposed *in vitro* to the action of undiluted sera from two different human donors and from two different rabbits. Figure 1 and Table I present the results of one such experiment with each of the five combinations (the letter "S" designates the sensitive and "R" the resistant strain). Results of duplicate experiments were very similar. Figure 1 shows the great difference in sensitivities of the two strains used in Combination I. Inspection of Table I reveals a similar pattern of results of experiments with the other four combinations. Where differences occur between the actions of rabbit and human sera, the latter are more active. Heated rabbit or human serum had no bactericidal activity against any strain.

Survival of serum-sensitive and serum-resistant gram-negative bacilli in vivo

Figure 2 and Table III present data from duplicate experiments performed with each of the five combinations and compare the survival rates in the blood stream of the sensitive and resistant strains of each combination after intravenous injection.

Combination I was composed of strains 124S and 118R. In Figure 2 it may be noted that the number of bacteria of each strain cultured from

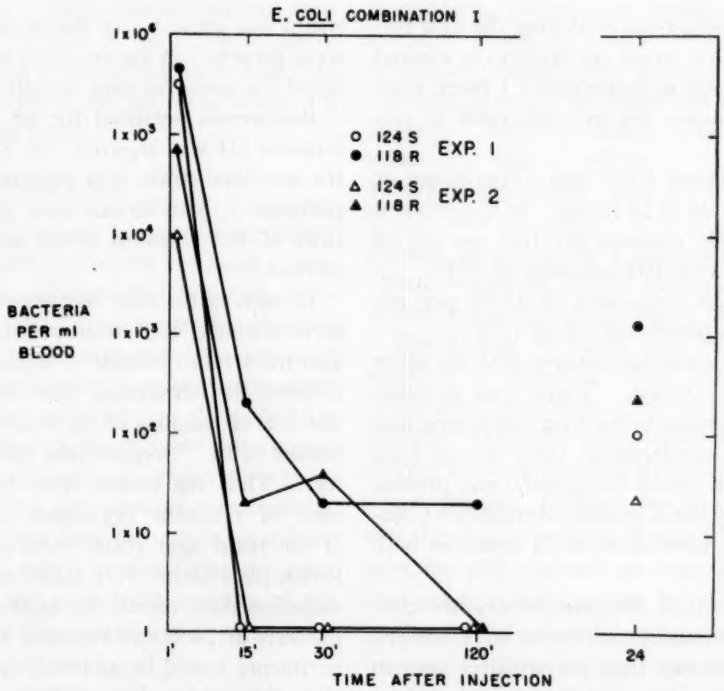

FIG. 2. NUMBER OF ORGANISMS OF EACH STRAIN OF COMBINATION I SURVIVING IN THE BLOOD STREAM OF THE RABBIT FOLLOWING INTRAVENOUS INJECTION

TABLE III

Rate of disappearance of bacterial strains in the blood stream of the rabbit following intravenous injection

Bacterial combination	Expt.	Bacterial strain	No. bacteria injected $\times 10^8$	No. bacteria per ml. blood at intervals after I.V. injection					
				1 min.	*15 min.*	*30 min.*	*120 min.*	*24 hr.*	*48 hr.*	*96 hr.*
II	1	*E. coli* 119R	5.2	79,000	100	0	20	0	0	0
		129S	3.6	16,000	0	0	20	0	0	0
	2	*E. coli* 119R	4.4	140,000	40	0	60	14	0	0
		129 S	5.1	55,000	100	0	0	0	0	0
III	1	*E. coli* 118R	2.8	670,000	200	20	40	576	42	0
		129S	1.8	85,000	20	0	0	8	0	0
	2	*E. coli* 118R	6.0	600,000	20	20	0	60	12	0
		129S	5.6	240,000	0	0	0	6	2	0
IV	1	*E. coli* 108R	3.9	1,900,000	48,000	240	100	264		
		130S	4.0	430,000	620	40	0	34		
	2	*E. coli* 108R	3.8	1,400,000	32,000	440	270	0	0	
		130S	3.0	21,000	40	20	40	0	0	
V	1	Aerobacter 125R	4.5	480,000	580	160	20	0	0	
		Klebsiella 27S	4.4	1,200,000	20	0	0	0	0	
	2	Aerobacter 125R	2.6	310,000	300	20	20	0	0	
		Klebsiella 27S	4.9	110,000	0	0	0	0	0	

the blood was rapidly reduced during the first two hours. The resistant strain appeared to be cleared at a slower rate, and in Experiment 1 there were 20 resistant organisms per ml. culturable at two hours.

In both experiments there was a continuing or recurring bacteremia at 24 hours. In Experiment 1, there were 1,240 colonies of 118R per ml. of blood compared with 104 colonies of 124S. In Experiment 2, 232 organisms of 118R per ml. were detected compared with 28 of 124S.

Table III reveals similar pictures with the other four combinations tested. There was a rapid clearing of both strains in the first two hours, and with each *E. coli* combination, there was at least one experiment in which bacteremia was present at 24 hours. Only the Klebsiella-Aerobacter Combination V was undetectable at 24 hours in both experiments.

If the data from all 10 experiments are considered, the serum-resistant strains were cleared at a slightly slower rate than the sensitive ones in nine experiments and at a similar rate in the other. In six of the experiments, bacteremia was present at 24 hours. In one of these six only the resistant strain was present. In the other five, both strains were present, but the resistant organisms outnumbered the sensitive ones usually about 10 to 1.

Bacteremia persisted for 48 hours after Combination III was injected. In Experiment 1, only the resistant strain was present, whereas in Experiment 2, both strains were present, but organisms of the resistant strain were the more numerous.

In each of the four combinations of *E. coli* described above, the strain resistant to serum was also the strain resistant to tetracycline. As noted *in vitro*, the tetracycline agar suppressed a certain number of colonies of each strain supposedly resistant to it. Streptomycin did not have this effect. Thus the counts from the experiments *in vivo* of resistant organisms are probably low. If the plain agar plate count minus the streptomycin plate count were taken rather than the tetracycline plate count, the number of resistant organisms at 24 hours reported for the *E. coli* experiments would be as much as doubled, but this alteration makes little difference to the general conclusions.

With each combination of *E. coli*, 5 to 15 colo-

nies which had appeared on the plain agar plate inoculated 24 hours after injection were picked and tested for their sensitivities to serum *in vitro*. Of the 63 tested, 61 were resistant to serum and two were sensitive. This ratio of 30 to 1 is not incompatible with that of 10 or 20 to 1 noted by the antibiotic marker technique.

Forty-three colonies obtained from the 24 hour blood cultures in tetracycline agar were tested for streptomycin sensitivity and serum resistance. All showed the same qualities as had the parent strain.

Twenty-eight colonies isolated from the blood 24 or 48 hours after the injection of Combinations I or III and representing the sensitive strains 124S and 129S showed the same sensitivity to human serum as before injection. In Combination III, cultures from four colonies of strain 129S from the 24 hour streptomycin plate showed the same degree of sensitivity when tested against two rabbit sera as the strain had shown before passage. Cultures from the two colonies on the streptomycin plate prepared 48 hours after injection showed the same degree of sensitivity to the infected rabbit's own serum taken at 48 hours as well as to serum from another rabbit. These data support the conclusions that only colonies of the sensitive strain were isolated on the streptomycin agar and that a change in sensitivity of the bacterial strain did not take place during passage.

DISCUSSION

Preliminary experiments showed that rabbit serum was bactericidal *in vitro* for the same strains as was human serum although its action was somewhat less pronounced. This same relationship between human and rabbit sera has been noted in experiments in which pooled sera were compared with respect to their action against *S. typhi* (5). As with the human sera, there were only minor differences among the rates at which sera from different rabbits killed any particular test strain. These results indicated that the rabbit would be a suitable animal in which to investigate the relative importance of the bactericidal activity of serum as a barrier to the invasion and persistence of bacteria in the blood stream.

The bacterial strains of each combination selected for the injection of the rabbits were vastly different in their degree of sensitivity to serum *in vitro*. Although in 9 out of the 10 experiments in the rabbit, the resistant strain disappeared from the blood stream at a less rapid rate than the sensitive one, the numbers of both strains were quickly diminished, and the differences in the rate of clearance between the two were unimpressive. If the resistant strains are considered absolutely resistant to serum as *E. coli* strain 108R appears to be, then it must be concluded that factors other than the bactericidal activity of serum play the major role in clearing the blood stream of bacteria. These results are in agreement with those of another study in which the injected strain of *E. coli* was known to be resistant to serum (6). That study, as well as a number of others (7–10), has shown quite clearly that the injected bacteria are removed as they pass through the liver and spleen, presumably by the reticuloendothelial system. A lesser number is removed during passage through the pulmonary circulation.

Despite the evidence above that the role played by the bactericidal property of serum is a minor one, the finding that in each of the six experiments in which bacteria were found at 24 hours following inoculation, the resistant strain outnumbered the sensitive strain ordinarily about 10 to 1, may adequately explain our observations that strains resistant to serum are more apt to appear in bacteremias. Bacterial counts of blood from patients bacteremic with *E. coli* or *S. typhi* rarely exceed 10 organisms per ml. and are usually less (11). Under such circumstances, it would seem that the chances of spread of a bacterium via the blood stream would be greatly enhanced if it were resistant to the bactericidal effect of serum.

The finding of viable bacteria of strains very sensitive to serum in blood cultures 24 and 48 hours after inoculation of the experimental animal indicates that the bactericidal action of serum *in vivo* is much less effective than testing *in vitro* would indicate. In these experiments it was shown that the strains isolated 24 and 48 hours following injection still retained the same sensitivity to human and to rabbit sera that had characterized them previously. Two of the isolates from a blood culture made 48 hours after infection were shown to be sensitive to the infected rabbit's own serum. These sensitive strains may have been protected by an intracellular locus or perhaps they were be-

ing fed continuously into the blood stream from cells of the reticuloendothelial system, and serum factors had not had time to affect them. The evidence for believing that the complement-dependent bactericidal system is relatively inactive *in vivo* has been reviewed by Maaløe (12).

SUMMARY

Rabbit serum was found to be bactericidal for strains of bacteria sensitive to human serum.

Pairs of enteric bacillary strains were injected intravenously into rabbits. One member of the pair was sensitive to the bactericidal action of serum, the other resistant. Five combinations of eight different strains were used and each combination was tested twice.

Both resistant and sensitive strains were rapidly cleared from the blood stream during the first two hours, the resistant strains being removed at a slightly slower rate in 9 of the 10 experiments.

Bacteremia was detected 24 hours following injection in six instances, and 48 hours after injection in two. Ordinarily, organisms of the resistant strain outnumbered those of the sensitive strain about 10 to 1. The bacteria of the sensitive strain which were isolated 24 or 48 hours after injection showed no change in their degree of sensitivity to the bactericidal action of serum.

It is concluded from these experiments that the bactericidal properties of serum play, at most, a minor role in freeing the blood stream of bacteria, but that, nevertheless, this role may be of some importance. This conclusion is reinforced by our previous observations that most spontaneously occurring bacteremias caused by gram-negative bacilli are caused by serum-resistant organisms.

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ALTERATIONS IN OSMOTIC AND MECHANICAL FRAGILITY RELATED TO *IN VIVO* ERYTHROCYTE AGING AND SPLENIC SEQUESTRATION IN HEREDITARY SPHEROCYTOSIS * †

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Increased osmotic and mechanical fragility of the erythrocytes is a consistent finding in patients with hereditary spherocytosis, although in a few individuals it is necessary to incubate the cells at 37° C. for 24 hours before the otherwise latent abnormality becomes demonstrable. Splenectomy will correct the hemolytic process of hereditary spherocytosis despite the continuing production of abnormally fragile cells by the patient. The spleen is, therefore, essential in the destruction of the intrinsically defective erythrocytes. In the peripheral blood of some individuals with this disease a "double population" of cells exists. One component consists of more markedly fragile cells that are not present after splenectomy and must, therefore, have been related to the presence of the spleen.

The present study was undertaken to investigate the changes in osmotic and mechanical fragility accompanying *in vivo* erythrocyte aging and splenic sequestration and to elucidate the source and fate of the more markedly fragile group of red cells found in some patients.

Advantage was taken of the increased erythropoiesis in patients with hereditary spherocytosis prior to splenectomy to label *in vivo* with Fe^{59} the new red cells produced during a short time span, thereby obtaining an identifiable group of cells of known age. Using techniques that will be described, it was possible to differentiate these isotopically-labeled cells from the other cells in the peripheral circulation and to compare their characteristics to those of the general population. The changes in osmotic and mechanical fragility occurring in these labeled red cells during *in vivo* aging and splenic sequestration were followed. In addition, erythrocytes were recovered from the spleen at the time of splenectomy and reinjected into the patient's peripheral circulation. The post-splenectomy survival and changes in osmotic and mechanical fragility of these cells, labeled with Cr^{51}, were followed and distinguished from the cells of the general population.

METHODS

Routine hematologic studies were performed by standard methods (1). Autohemolysis was determined by the method of Selwyn and Dacie (2) as modified by Young, Izzo, Altman and Swisher (3). Erythrocyte osmotic fragility tests were done as described by Emerson and associates (4) and the osmotic fragility of the labeled cells was distinguished from that of the general population by the following modification of this method. The volume of each saline solution was increased to 3 ml., and 0.3 ml. of defibrinated whole blood was added to each tube. These were then centrifuged, decanted and the hemoglobin content of 0.5 ml. of the supernatant solution determined in a Beckman (Model B) spectrophotometer. The remainder of the supernatant was transferred to a calibrated isotope counting tube and the radioactivity determined in a low background, well-type scintillation counter (Tracerlab). Sufficient counts were obtained to give a statistical error of ± 3 per cent or less. As a blank, 0.3 ml. of the defibrinated whole blood was suspended in hypertonic saline (1.25 Gm. NaCl per 100 ml.) and the radioactivity found in this supernatant after centrifugation was subtracted from that obtained for the other samples. The value for radioactivity thus obtained represented isotope released by the red cells lysed at each concentration of sodium chloride. The amount of radioactivity in each sample was calculated as a percentage of that released into the supernatant when 0.3 ml. of the defibrinated whole blood had undergone complete hemolysis by addition to 3 ml. distilled water. The results were plotted as two curves, one showing the percentage of hemolysis measured as hemoglobin (the osmotic fragility of all the cells in the sample) and the

* A preliminary report of this work appeared in abstract form (J. clin. Invest. 1958, 37, 899).

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other curve representing the percentage of radioactivity released at each concentration of sodium chloride (the osmotic fragility of only the isotopically labeled cells). Control studies demonstrated that no appreciable Fe^{59} was present in the stroma of the lysed cells and that osmotic fragility curves obtained by this isotope method on our patients after general distribution of the Fe^{59} through the red cell population were identical with those obtained by the usual method in which hemoglobin from lysed red cells is measured.

The mechanical fragility of the red cells was determined by a modification of the method of Shen, Castle and Fleming (5). The hematocrit of a sample of defibrinated venous blood was adjusted to 35 per cent and 0.5 ml. subjected to the standardized trauma produced by rotating it for 90 minutes in a 50 ml. Erlenmeyer flask at 30 rpm with 10 uniform 4 mm. selected glass beads. To obtain an adequate sample for the isotope counting procedures, two flasks were used for each determination and combined after rotation. The results were corrected by a blank consisting of a similar blood sample in hypertonic saline (1.25 Gm. NaCl per 100 ml.) and expressed as percentage of hemolysis compared to hemolysis in distilled water.

Ten μc. of Fe^{59} in the form of ferric chloride (Abbott) (3 to 10 μg. of elemental iron) was incubated with 20 ml. of normal compatible heparinized plasma and administered intravenously to each patient. Since patients with hemolytic anemias have increased saturation of their iron-binding protein, normal compatible plasma was used instead of the patient's. The plasma Fe^{59} clearance and the red cell Fe^{59} utilization studies were performed according to the method of Huff and associates (6, 7).

Red cell survival was determined by the method of Ebaugh, Emerson and Ross (8) using 120 μc. of Cr^{51}. In each case the patient's own red cells, obtained from either peripheral vein or splenic pulp, were used. Intact red cells were recovered from the spleen at operation. Using sterile technique, this was accomplished by making a number of large incisions into the surgical specimen immediately after removal and allowing the formerly entrapped blood to flow into a glass flask where it was defibrinated by rotating with glass beads.

Scintillation counting over the body surface was done by the methods described by Huff and co-workers (7) and Jandl, Greenberg, Yonemoto and Castle (9). The counting rates over the liver and spleen are plotted as ratios relative to the counting rate over the precordium.

RESULTS

Three unrelated adults who were referred to this hospital for investigation of anemia or jaundice were studied. All had splenomegaly and showed spherocytosis on peripheral blood smears; appropriate laboratory tests confirmed the diagnosis of hereditary spherocytosis. Family history in all three was negative for anemia, jaundice or splenomegaly. The mother of Case 2 was the only relative available for examination and her blood studies, including osmotic and mechanical fragility, were normal. Identifying data and laboratory findings are recorded in Table I. Presplenectomy studies with Fe^{59} were performed on Cases 1 and 2 and post-splenectomy studies with Cr^{51} on Cases 2 and 3.

Presplenectomy studies

Case 1 showed evidence of a marked hemolytic process. A Cr^{51} autosurvival had been performed in our laboratory on this individual one year prior to the present study and demonstrated a red cell half-life of eight days compared to a normal of at least 28 days for this method. During the present study he showed a marked hyperbilirubinemia (indirect reacting) and sustained reticulocytosis (Table I). Both the spleen and liver were enlarged. The administered Fe^{59} was rapidly cleared from the plasma, with a half-time of 15 minutes, contrasted to a normal of 60 to 120 minutes. The utilization of Fe^{59} for red cell formation and the

TABLE I

Laboratory data on patients in study

Case	Age Race Sex	Hgb.*	Hct.	Retics.	Bilirubin direct	Bilirubin total	Erythrocyte osmotic fragility	Erythrocyte mechanical fragility 0 hrs.	Erythrocyte mechanical fragility 24 hrs.	% Autohemolysis 48 hours No glucose	% Autohemolysis 48 hours With glucose	Plasma clearance Fe^{59} half-time	Erythrocyte survival Cr^{51} half-time
		Gm.%	*%*	*%*	*mg./100 ml.*			*%*	*%*			*min.*	*days*
Normal range								1–5	9–15	0–3	0–1	60–120	28+
1	68 W M	10	30	15	1.4	5.1	Increased	22	37			15	8
2	32 N F	9.5	32	4	0.7	1.1	Increased	18	37	12.4	8.3	31	
3	54 W M	9.5	29	3	0.5	1.0	Increased	7	17	5.7	1.5		16

* Hgb. = hemoglobin; Hct. = hematocrit; Retics. = reticulocytes.

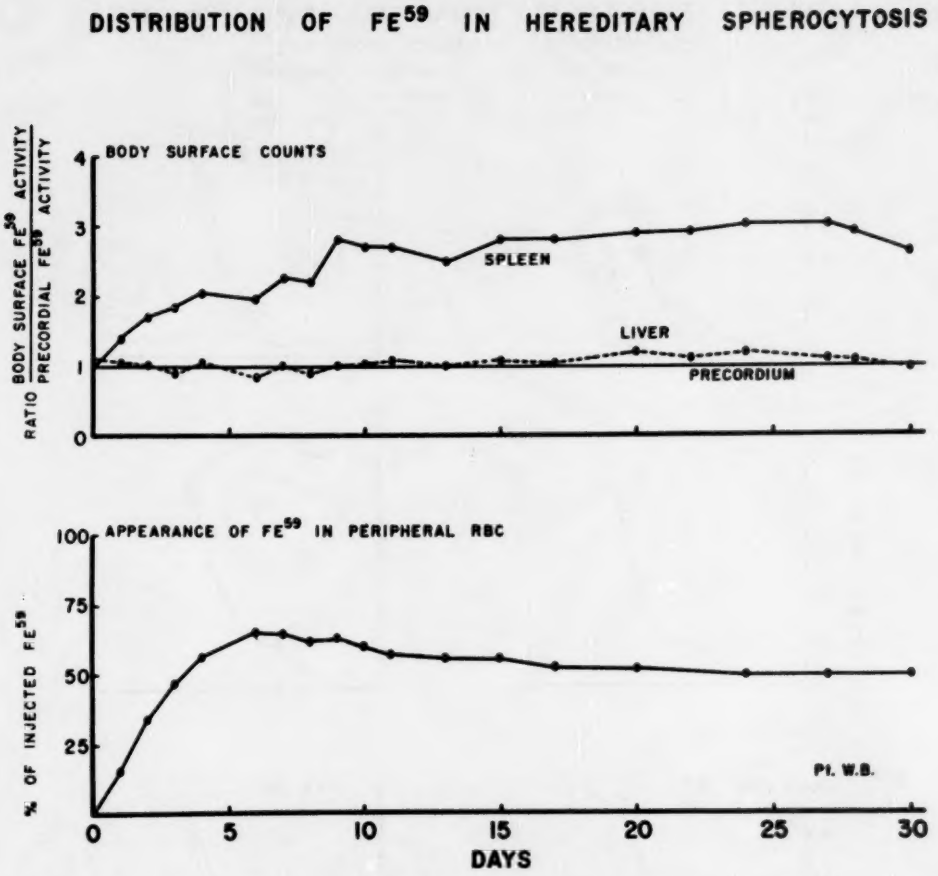

FIG. 1. CASE 1: UTILIZATION OF Fe^{59} FOR RED CELL FORMATION AND *IN VIVO* DISTRIBUTION

distribution of body surface counts are represented in Figure 1. There was rapid incorporation of Fe^{59} into new red cells as might be anticipated in a patient with hemolytic anemia. This reached a maximum of 65 per cent on Day 6. Body surface counting demonstrated early and rapid accumulation of isotope in the spleen after the expected initial rise and fall in counting rate over the sacral bone marrow.

Peripheral blood samples were studied at frequent intervals for changes in erythrocyte fragility by the osmotic and mechanical fragility methods described above. There was not sufficient radioactivity present in the red cells prior to the third day to give statistically valid counting rates. The results of the studies of the osmotic fragility on Days 3, 7, 10, 13, 24 and 30 are presented in Figure 2. The osmotic fragility of the patient's total peripheral red cell population is increased. The lower portion of the fragility curve shows some asymmetry with approximately 20 per cent of the cells being more markedly fragile than the other red cells in the peripheral blood. The broken lines represent the osmotic fragility of the Fe^{59}-labeled red cells. Since the labeling of the red cells is occurring as they are formed *in vivo,* on Day 3 all cells represented by this curve must be no more than three days old. The fragility curves of these new cells on Days 3 and 7 show some increase in fragility as compared to normal red cells, but do not have the asymmetrical curve of the total peripheral cell population and none of the cells are as osmotically fragile as some of the red cells in the peripheral blood. It is not until Day 10 and subsequently that some of the labeled cells show fragility changes comparable to that of the general cell population.

During the early phases of this study, observa-

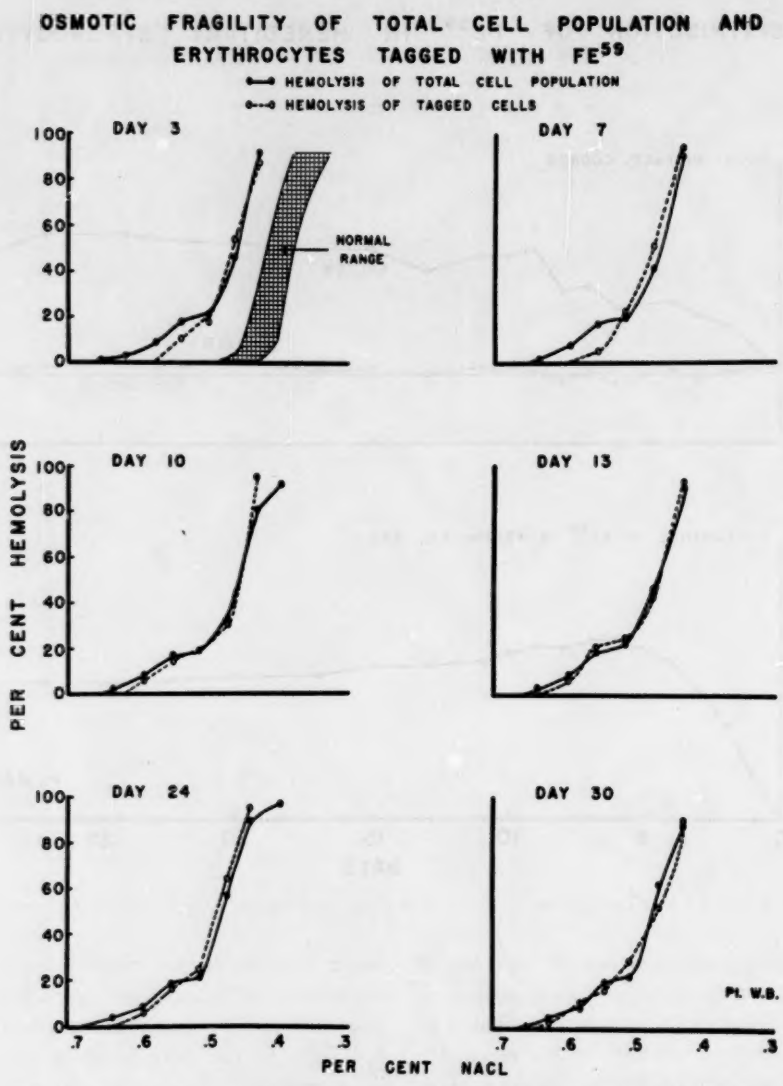

FIG. 2. CASE 1: OSMOTIC FRAGILITY OF THE Fe^{59}-LABELED RED CELLS CONTRASTED WITH THAT OF THE TOTAL PERIPHERAL CELL POPULATION

tions were also made on cells after sterile incubation for 24 hours. However, no difference was noted between the fragility of the labeled cells and the general peripheral population, and these studies were not continued. All observations reported are on fresh, unincubated whole blood.

The concurrent changes in mechanical fragility of red cells of Case 1 are depicted on the lower half of Figure 3. The mechanical fragility of this patient's peripheral red cells varied from 18 to 27 per cent compared to a normal of 1 to 3 per cent by this method. On Day 3 the mechanical fragility of the new red cells as measured by the Fe^{59} was 10 per cent. This gradually increased until it was equal to that of the total cell population (24 per cent) on Day 13. Subsequently, the labeled cells exhibited a mechanical fragility slightly increased above that of the general population.

A similar study was performed on Case 2 and the results are outlined in Figures 3 through 5. This individual showed evidence of a less severe hemolytic process, a reticulocytosis of 4 per cent

and no hyperbilirubinemia (Table I). The half-time for plasma clearance of Fe^{59} was 31 minutes with rapid reappearance of the isotope in the peripheral red cells. This reached a maximum recorded utilization of 80 per cent on Day 10. There was evidence by body surface counting of gradual accumulation of Fe^{59} in the spleen (Figure 4). Studies of osmotic fragility similar to those described above were performed on this patient and the results are presented in Figure 5. In an effort to diminish the reutilization of Fe^{59} from destroyed red cells in further red cell production, a relatively large dose of nonisotopic iron was administered beginning on Day 11 of the study. From Day 11 to Day 28, 950 mg. of iron was administered in divided doses intramuscularly in the form of Imferon®. Approximately 15 per cent of this patient's peripheral red cells showed a marked increase in osmotic fragility as represented by the lower portion of the curve (solid lines, Figure 5). On Day 4 the new Fe^{59}-labeled red cells, represented by the broken line, did not show any cells with this marked increase in fragility. By Day 11 the proportion of fragile cells had increased and by Day 17 the proportion had increased above that of the general peripheral cell population and about 20 per cent of the cells showed a marked increase in fragility. These very fragile cells then apparently disappeared from the peripheral circulation within several days, for the fragility curve of the labeled cells on Day 24 is similar to that seen on Day 4. Subsequent studies, not shown in the figures, demonstrated that a portion of the labeled cells again became more fragile and both curves were essentially the same after Day 31.

The results of the mechanical fragility studies on this patient are presented in the upper half of Figure 3. As in the previous study, the mechanical fragility of the labeled cells was initially low and then gradually increased, eventually becoming slightly greater than that of the general cell population.

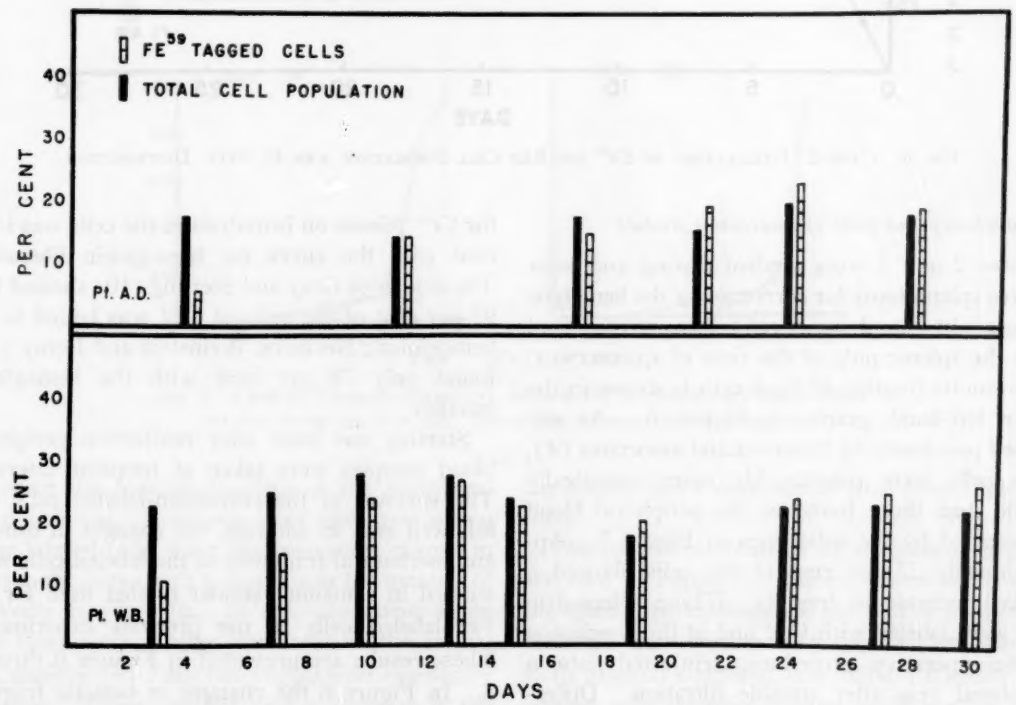

FIG. 3. MECHANICAL FRAGILITY OF THE Fe^{59}-LABELED CELLS CONTRASTED WITH THAT OF THE TOTAL PERIPHERAL CELL POPULATION IN CASES 1 (W. B.) AND 2 (A. D.)

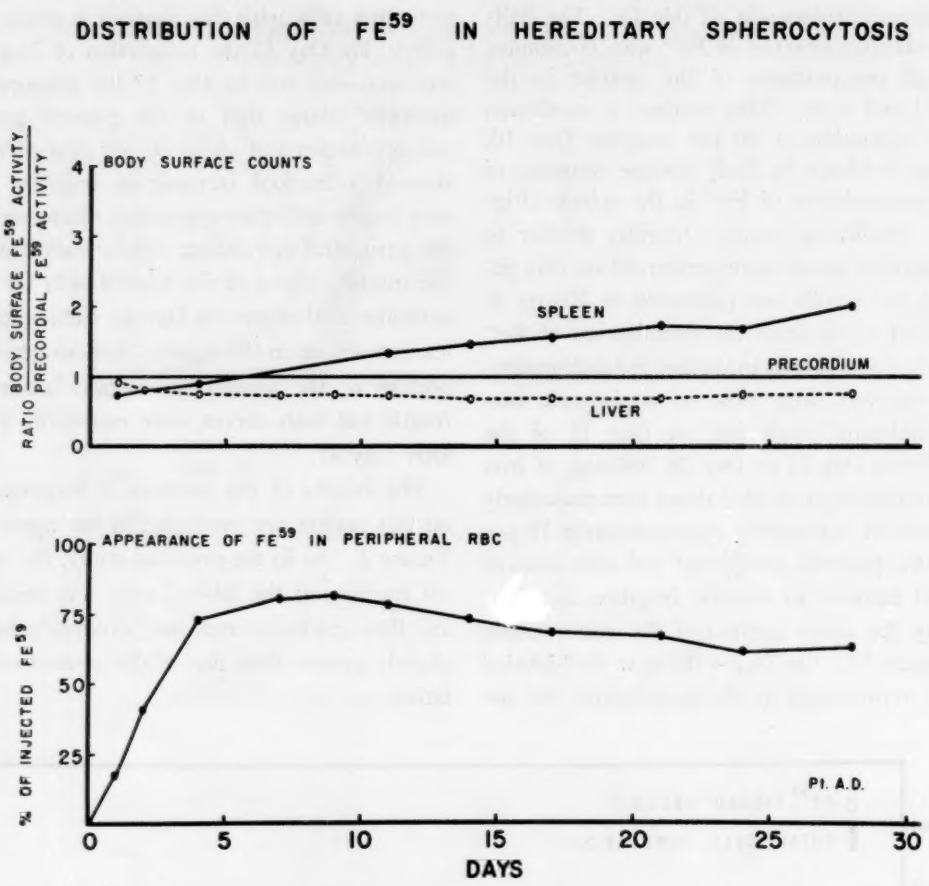

FIG. 4. CASE 2: UTILIZATION OF Fe^{59} FOR RED CELL FORMATION AND *IN VIVO* DISTRIBUTION

Splenectomy and post-splenectomy studies

Cases 2 and 3 were studied during and after elective splenectomy for correction of the hemolytic process. In Case 2, intact red cells were recovered from the splenic pulp at the time of splenectomy. The osmotic fragility of these cells is shown in the upper left-hand graph of Figure 6. As described previously by Emerson and associates (4), these cells were considerably more osmotically fragile than those found in the peripheral blood represented by the solid lines in Figure 5. Approximately 25 per cent of the cells showed a marked increase in fragility. These spleen-drip cells were labeled with Cr^{51} and at the conclusion of the operative procedure, reinjected into a peripheral vein after suitable filtration. Differential osmotic and mechanical fragility studies of a small sample of these cells indicated that the curve for Cr^{51} release on hemolysis of the cells was identical with the curve for hemoglobin liberation. The studies of Gray and Sterling (10) showed that 95 per cent of the red cell Cr^{51} was bound to the hemoglobin; Necheles, Weinstein and Leroy (11) found only 78 per cent with the hemoglobin fraction.

Starting one hour after reinjection peripheral blood samples were taken at frequent intervals. The survival of the chromium-labeled cells was followed and, in addition, the changes in osmotic and mechanical fragilities of the labeled cells were studied in a manner similar to that used for the Fe^{59}-labeled cells in the previous experiments. These results are presented in Figures 6 through 8. In Figure 6 the changes in osmotic fragility are recorded. The broken lines represent the isotope-labeled, spleen-drip cells and the solid lines

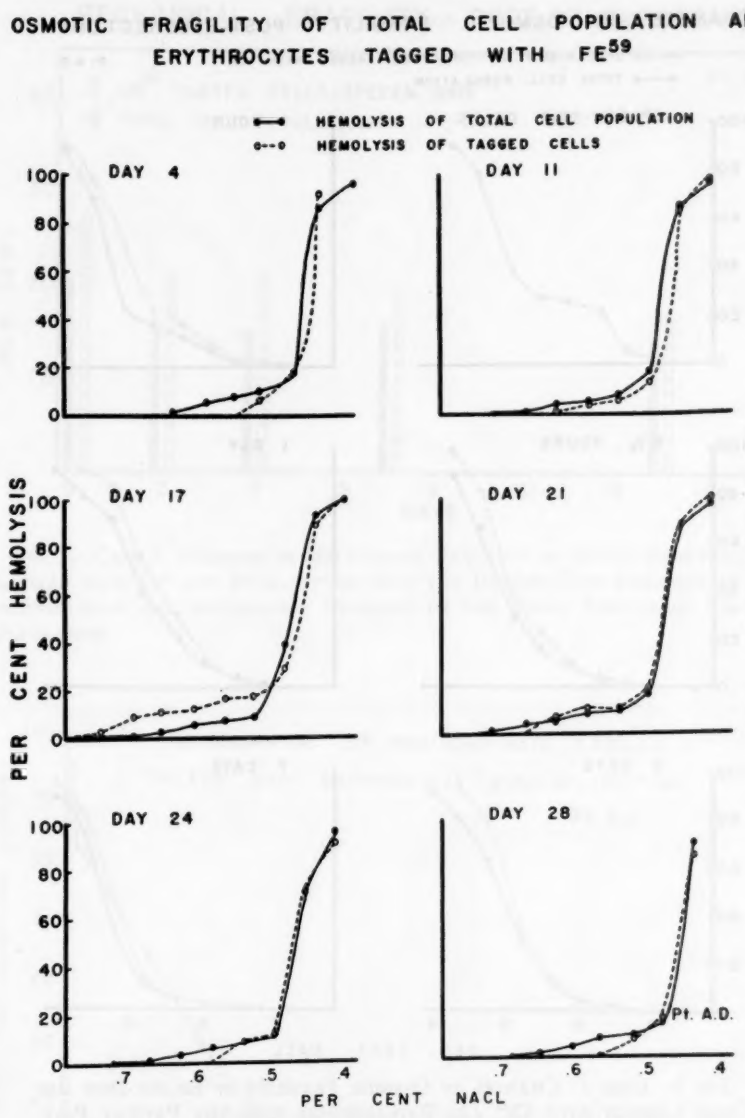

FIG. 5. CASE 2: OSMOTIC FRAGILITY OF THE Fe^{59}-LABELED RED CELLS CONTRASTED WITH THAT OF THE TOTAL PERIPHERAL CELL POPULATION

represent the osmotic fragility of the peripheral cell population. Even one hour after reinjection of the labeled cells, there has been some change in the osmotic curve with a decrease in the number of markedly fragile cells. In the subsequent seven days there were further decreases in the fragile cells, until by Day 7 the two curves were essentially the same. There also has been a decrease in the number of markedly fragile cells in the peripheral blood as a whole, so that the curve of hemolysis has become more symmetrical than before splenectomy.

The concurrent changes in mechanical fragility are seen in Figure 7. Twenty-seven per cent of the spleen-drip cells and 20 per cent of the peripheral cells were destroyed by the standard trauma. Both gradually showed less marked fragility, the labeled cells showing a more rapid change, both mechanical fragilities being essentially the same by Day 13.

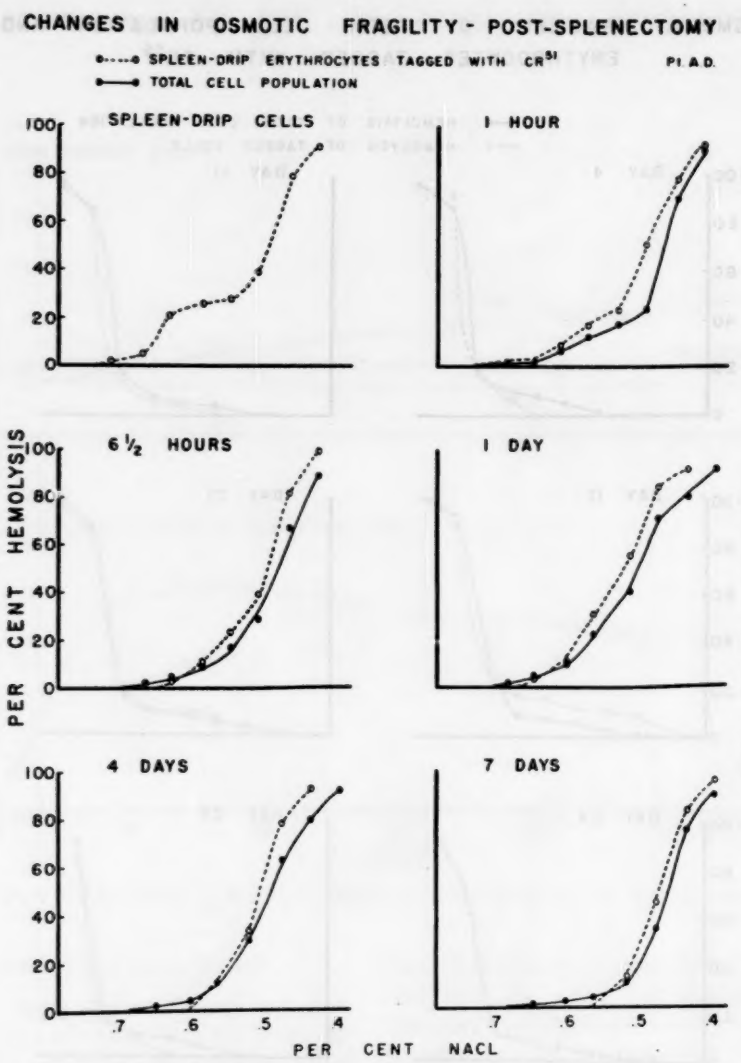

FIG. 6. CASE 2: CHANGES IN OSMOTIC FRAGILITY OF SPLEEN-DRIP RED CELLS LABELED WITH Cr^{51} AND RETRANSFUSED INTO THE PATIENT POST-SPLENECTOMY CONTRASTED WITH THE OSMOTIC FRAGILITY OF THE TOTAL PERIPHERAL RED CELL POPULATION

The survival of these Cr^{51}-labeled, spleen-drip red cells is presented in Figure 8. The one hour postinjection sample, the first one obtained, was plotted as 100 per cent. During the first 48 hours there was a rapid loss of approximately 20 per cent of the labeled cells. The remainder of the cells showed a normal length of survival.

A similar study of the postsplenectomy survival of spleen-drip red cells was performed on Case 3 and the findings recorded in Figure 9. The solid line represents the Cr^{51} autosurvival of this patient's red cells done in our laboratory several months prior to splenectomy. A half-life of 16 days was obtained. A sample of cells obtained from the spleen at the time of splenectomy was labeled with Cr^{51} and reinjected into the peripheral circulation postoperatively. About 30 per cent of these cells showed a marked increase in osmotic fragility. The survival curve (broken line, Figure 9) reveals that 50 per cent of the autotrans-

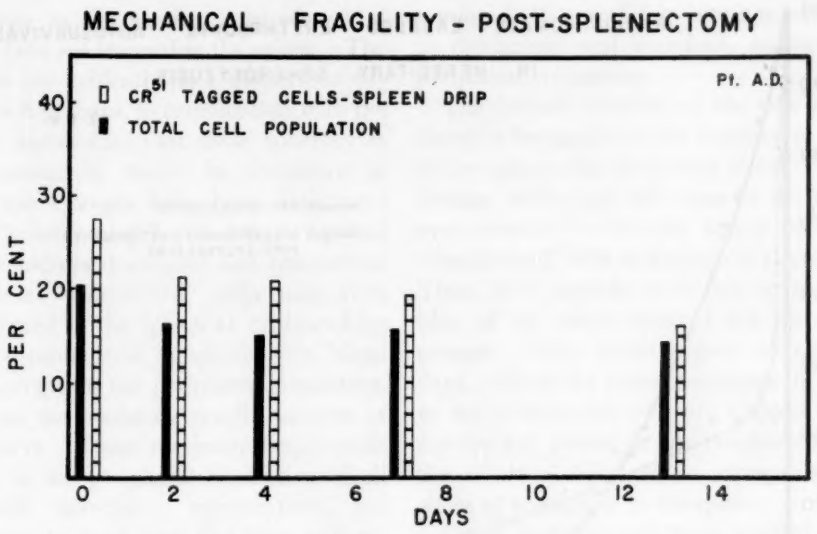

FIG. 7. CASE 2: CHANGES IN MECHANICAL FRAGILITY OF SPLEEN-DRIP RED CELLS LABELED WITH Cr^{51} AND RETRANSFUSED INTO THE PATIENT POST-SPLENECTOMY CONTRASTED WITH THE MECHANICAL FRAGILITY OF THE TOTAL PERIPHERAL RED CELL POPULATION

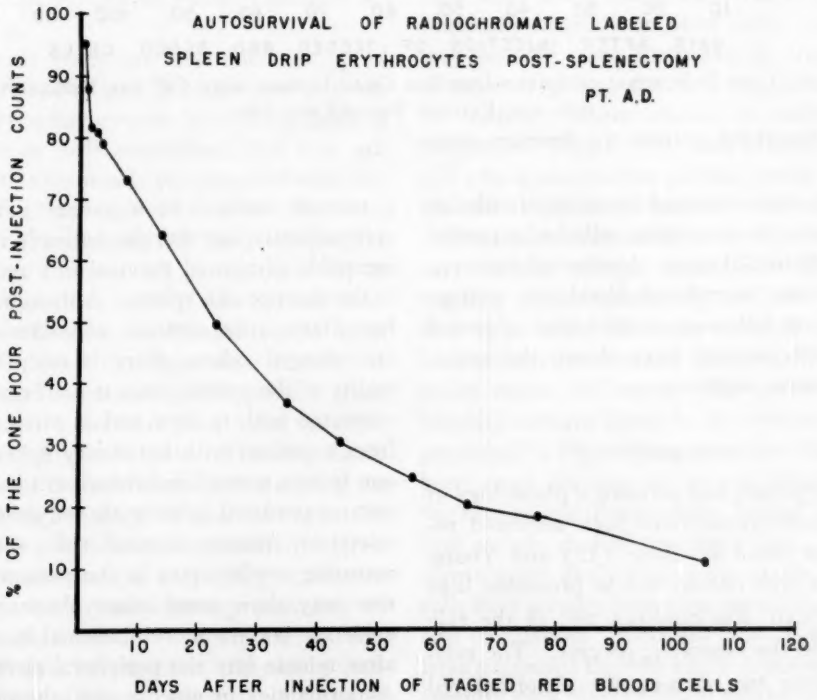

FIG. 8. CASE 2: SURVIVAL OF SPLEEN-DRIP RED CELLS LABELED WITH Cr^{51} AND RETRANSFUSED INTO THE PATIENT POST-SPLENECTOMY

Data are not corrected for chromium elution.

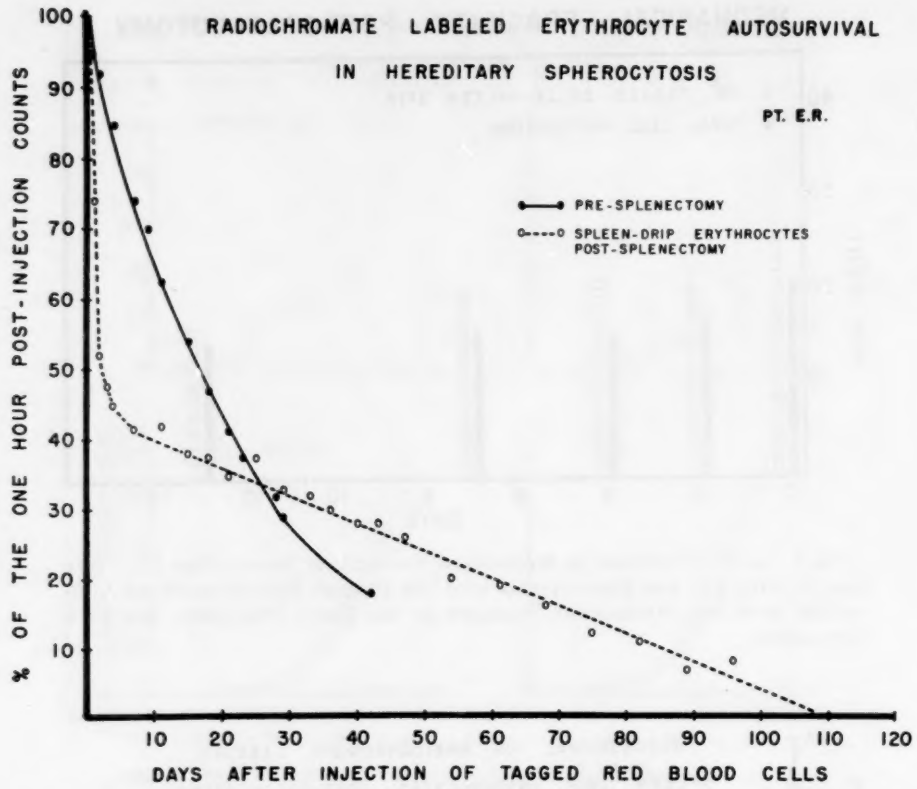

FIG. 9. CASE 3: SURVIVAL OF SPLEEN-DRIP RED CELLS LABELED WITH Cr^{51} AND RETRANSFUSED INTO THE PATIENT POST-SPLENECTOMY

Data are not corrected for chromium elution.

fused red cells were removed from the circulation within 48 hours; the remaining cells had a normal life span of 100 to 120 days. Neither of these patients showed any significant blood loss postoperatively and on follow-up studies over a period of months, both patients have shown the anticipated good clinical response.

DISCUSSION

The clinical picture and pathologic physiology of hereditary spherocytosis have been reviewed recently in some detail by Dacie (12) and Young (13). Only a brief outline will be presented here of the erythrocyte abnormalities and of the role of the spleen in the hemolytic process. The relationship of these two factors, the abnormal red cell and the spleen, has been most concisely demonstrated in a study of red cell survival by Emerson (14). He showed that normal red cells had a normal survival in a patient with hereditary spherocytosis, but that the patient's red cells were incapable of normal survival in a recipient *except* in the absence of a spleen. Although patients with hereditary spherocytosis characteristically have an enlarged spleen, there is no primary abnormality of the spleen, since it has been amply demonstrated both *in vivo* and *in vitro* that a spleen from a patient with hereditary spherocytosis and one from a normal individual will both selectively remove perfused spherocytic red cells but will not selectively remove normal red cells. Although maturing erythrocytes in the patient's bone marrow may show some minor shape changes, they evidently become more abnormal in shape shortly after release into the peripheral circulation (12). Abnormalities in glucose and phosphate metabolism have been demonstrated in these cells (15); but probably related to the abnormal thickness of the cells and their inability to escape easily through

slit-like openings in the venous sinusoids (12), the spheroidal cells are trapped in the spleen. The entrapped cells are evidently in a deleterious environment, which perhaps, in combination with the deficiencies of metabolism that these spherocytes demonstrate, eventually results in alterations in the cells. These changes have been designated by the term "conditioning," and are recognized by abnormally increased osmotic and mechanical fragility. These "conditioned" cells may then either be destroyed in the spleen as evidenced by the elevated bilirubin level in splenic vein blood (12), or, escaping into the peripheral circulation, be identified as the markedly fragile portion of the osmotic curve. These markedly fragile cells are not found in the peripheral circulation of all individuals with hereditary spherocytosis, but their relationship to the spleen has been well established by the experiments of Young, Platzer, Ervin and Izzo (16), Dacie (12), Emerson and associates (4), and Weisman, Ham, Hinz and Harris (17), who have shown that fragile cells of this type are found in the spleen pulp and they are not present in the peripheral blood of patients after splenectomy.

In the present study an identifiable group of spherocytic red cells was produced in patients with hereditary spherocytosis by *in vivo* labeling with Fe^{59}. It is well established that iron administered in this fashion is incorporated only into new red cells and not into older cells. However, when the red cell containing Fe^{59} is destroyed, the iron is reutilized for new cell production. In an individual with normal red cell survival and normal iron metabolism, no reutilization would occur for 100 to 120 days. Red cells were rapidly being destroyed in the patients in the present study so that Fe^{59} was, to some degree, being incorporated repeatedly into new cells. This fact limits the usefulness of this technique for studying the cells for a prolonged period of time. Despite this objection it was possible to demonstrate progressive and significant changes in fragility of the new red cells and to follow them for approximately 30 days. Estimation of Fe^{59} incorporation into peripheral red cells by the usual techniques indicated a utilization of 65 per cent in Case 1 and 80 per cent in Case 2. This undoubtedly does not represent the true maximum utilization of the isotope for hemoglobin labeling since red cells containing Fe^{59} were being continuously sequestered in the spleen and therefore, removed from the peripheral circulation.

The osmotic fragility of the new red cells was found to be similar to the fragility of the majority of the cells in the peripheral blood. Of particular interest is the fact that none of the new erythrocytes was as osmotically fragile as some of the "conditioned" cells in the patient's peripheral blood. Thus, it is possible from this study to get some idea of the time required for the conditioning process. This would appear to be at least 10 days. From the present evidence it is impossible to say whether the cells are trapped in the spleen for the full period or the conditioning process is the result of recirculation or repeated short episodes of stagnation in the spleen.

Other investigators have studied the changes in red cell fragilities of dogs and humans without hematologic disease. Cruz, Hahn, Bale and Balfour (18), and later, Stewart, Stewart, Izzo and Young (19) using dog erythrocytes labeled *in vivo* with radioactive iron, found that newly formed cells were more osmotically fragile and less mechanically fragile than older cells. As they aged, they became more mechanically fragile, but the osmotic fragility was not significantly altered. In contrast, recent studies in normal man by Simon and Topper (20) and Marks and Johnson (21) have shown that normal young erythrocytes are more resistant to osmotic lysis than older cells.

The observations made after splenectomy on the survival and changes in osmotic and mechanical fragility of the spleen-drip red cells demonstrate that cells with the most marked abnormalities have a short survival and disappear from the circulation within 48 hours. The curves for osmotic fragility changes found in the spleen-drip red cells presented in Figure 6 suggest that there may have been some decrease in osmotic fragility between the time of reinjection of the labeled blood and the first sample studied one hour later. However, it seems more likely that some of the most fragile cells have already been eliminated from the peripheral circulation. Motulsky and co-workers (22) have suggested that the marked increase in osmotic fragility may revert toward normal if the cells are placed in a circulation without a spleen. He transfused red cells labeled with Cr^{51} from a patient with hereditary spherocytosis, who had not

undergone splenectomy and had a cell population consisting of approximately 30 per cent very fragile cells, to the patient's splenectomized brother whose peripheral blood had no very fragile cells. The labeled donor cells showed a gradual decrease in osmotic fragility over a six day period. Since information on survival of these transfused cells is not presented, the observed changes could be due either to a reversible process in the very fragile red cells or to destruction and removal of these cells from the circulation. There is the possibility that the spleen-drip red cells studied in the present experiments had irreversible changes in contrast to the peripheral blood cells studied by Motulsky. He also performed the reverse experiment and transfused labeled red cells from the patient without a spleen into the brother with a spleen. Approximately six days were required for these red cells to show the marked increase in fragility seen in the recipient's own cell population.

The data from the present study demonstrate that in some patients with hereditary spherocytosis there is a progressive increase in osmotic and mechanical fragility of a portion of the red cell population, and that a period of approximately 10 days is required for these changes to reach their maximum from the time the cell first appears in the peripheral circulation. Previous information and the present study certainly implicate the spleen as the source of these very fragile red cells, since they can be found in the splenic pulp and are no longer seen in the peripheral blood after removal of the spleen. From information available at the present time it is impossible to say just how these red cells are conditioned in the spleen. Our experiments on the survival of red cells obtained from the spleen indicate that these very fragile cells have a short life span in the peripheral circulation, less than 48 hours. There was no evidence that the osmotic fragility of these cells reverted toward normal in the absence of the spleen. Therefore, it seems likely that in some patients with hereditary spherocytosis a significant proportion of the red cells is conditioned in the spleen over a period of days and escapes into the peripheral circulation, there undergoing rapid destruction either by the forces of mechanical trauma, osmotic lysis, or undetermined mechanisms.

SUMMARY

Changes in erythrocyte osmotic and mechanical fragility associated with aging and splenic sequestration were studied in two patients with hereditary spherocytosis by the use of Fe^{59}. New red cells showed some increase in fragility, but after approximately 10 days, conditioned cells with a marked increase in osmotic and mechanical fragility appeared in the peripheral circulation. At the time of splenectomy, red cells were recovered from the spleen, labeled with Cr^{51} and reinjected into the patient's peripheral circulation. The conditioned, most fragile cells disappeared rapidly, surviving less than two days; the other red cells, less osmotically and mechanically fragile, had a normal length of survival.

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THE ROLE OF THE KIDNEY IN ERYTHROPOIESIS*

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The pathogenesis of anemia in chronic and acute renal insufficiency has not yet been fully understood. Emerson and Burrows (1) and Chaplin and Mollison (2), using the Ashby technic, have shown the decreased red cell survival in chronic and acute nephritis. Later other workers (3–6) noted the same defect, and Muirhead and co-workers (7, 8) studying nephrectomized rabbits and dogs pointed out a hemolytic picture. Loge, Lange and Moore (9) in 1950, using radioactive iron, observed a reduced uptake in the red cells of uremic patients; these findings were confirmed by others using Fe^{59} with high specificity (4–6, 10). This reduction of the erythropoietic function was also demonstrated by the depletion of erythroblasts from the marrow in uremic patients (6, 11, 12) and by the severe erythroblastopenia commonly associated with acute renal failure (6, 13). These observations show that uremic anemia results from a double mechanism: a short red cell survival and a depression of erythropoiesis.

It was postulated without evidence that toxic products resulting from renal failure impaired marrow red cell production. This view was supported by *in vitro* experiments; Sacchetti (14) observed reduction of mitosis and Markson and Rennie (15) reported inhibition of erythroblast maturation after adding uremic plasma to marrow culture.

Recently Jacobson, Goldwasser, Fried and Plzak (16) showed the absence of erythropoietic factor response in hypoxic nephrectomized rats and assumed that the kidney was the source of an erythropoietic factor.

In preliminary notes (17–19) we reported that bilateral nephrectomy of dogs abolished erythropoiesis while bilateral ureter ligation did not impair this function despite the same elevation in blood urea nitrogen and the same degree of malnutrition. Details of these experiments and further data are described in this paper.

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MATERIALS AND METHODS

Forty-four mongrel dogs weighing between 15 and 25 Kg. were used. Fifteen dogs were subjected to bilateral nephrectomy and nine to bilateral ureter ligation or unilateral nephrectomy with ureter ligation of the opposite kidney. One dog was submitted to unilateral uretral ligation and, 15 days later, to removal of the opposite normal kidney. The controls consisted of nine normal dogs and three unilaterally nephrectomized dogs.

Nephrectomy and ureter ligation were done by lumbar approach. Ureters were ligated and cut. Bilateral nephrectomy and ureter ligation after unilateral nephrectomy were done in two steps in order to avoid fatalities; the second operation was carried out 15 to 30 days after the first one. Unilateral ureter ligation following a previous nephrectomy was preferred to bilateral ureter ligation after observation that the first procedure reduced mortality and that unilaterally nephrectomized dogs had a normal erythropoiesis. For convenience, no distinction will be made among dogs submitted to ureter ligation by one or the other procedure.

The uremic dogs were maintained for 7 to 15 days by peritoneal lavages according to Grollman, Turner and McLean's procedure (20). Lavages were started 72 hours after the second nephrectomy or ureter ligation.

Generally, after the second nephrectomy or ureter ligation the dogs refused food; no attempt was made to feed them. Two dogs were bled before the second nephrectomy; one was bled 200 cc. (1.5 per cent body weight) the day before nephrectomy; the other was subjected to three 200 cc. bleedings, during the week before the operation. Two dogs were severely bled (2.5 per cent body weight blood was drawn every day) immediately after removal of the second kidney. One dog was bled (1.5 per cent body weight blood withdrawn during four consecutive days) immediately after ureter ligation.

Bone marrow was drawn regularly by iliac crest puncture and stained by May-Grunewald-Giesma: erythroblasts were counted on 500 or 1,000 cells. Urea was measured by the urease technic (21). Potassium determinations were performed by flame photometer technic (22). Hematocrit and reticulocyte counts were determined every other day.

Iron metabolism studies were performed by the following methods. Plasma iron determinations were performed by the method of Hamilton, Gubler, Cartwright and Wintrobe (23). Blood volume was assumed to equal 8 per cent of body weight. Disappearance of Fe^{59} from the plasma, iron turnover and red cell uptake of Fe^{59}

were determined by the method of Huff and associates (24).

Plasma Fe^{59} disappearance and iron turnover were carried out 72 hours after second nephrectomy or ureter ligation; red cell incorporation of Fe^{59} was measured during the following days. Liver and spleen radioactivity was counted on tissue samples taken after death of the dog.

In an attempt to investigate marrow responsiveness to the erythropoietic factor in the uremic state,[1] one dog (H) was injected with a potent erythropoietic factor of human origin [2] before and after nephrectomy. This factor was prepared by collodion adsorption from the urine of an anemic patient (25). Injection of this active material into rats, 1 mg. daily during 14 days, resulted in a 40 per cent increase of the red cell volume (26). Iron measurements and marrow study were carried out during three periods: first period, normal dog under control conditions; second period, in the same dog, Fe^{59} was injected 24 hours after two successive daily I.V. administrations of 150 mg. of the erythropoietic factor; third period in the same dog, Fe^{59} was injected 24 hours after two successive daily I.V. administrations of 150 mg. of the erythropoietic factor which were begun 24 hours after the second nephrectomy.

It has not been possible to repeat this experiment which needed a very large amount of erythropoietic factor.

RESULTS

1. *Urea (Figures 1 and 2) and potassium measurements*

Urea and potassium plasma level was similar in both bilaterally nephrectomized dogs and ureter-ligated dogs. Three days after the second nephrectomy, which was the time of Fe^{59} injection, urea level was between 235 and 338 mg. per 100 ml. and potassium between 5.5 and 7.6 mEq. per L.; six days after nephrectomy, urea level was 240 to 532 mg. per 100 ml. and potassium was 6 to 8.7 mEq. per L. Three days after ureteral ligation, urea level was between 217 and 350 mg. per 100 ml. and potassium between 5.1 and 6.7 mEq. per L. Six days after ligation urea level was from 287 to 448 mg. per 100 ml. and potassium from 7.1 to 8.8 mEq. per L.

2. *Modifications of marrow erythroblasts*

After unilateral nephrectomy. Marrow of 13 dogs was investigated after unilateral nephrectomy and disclosed a decrease of normoblasts; after initial lowering, return to higher values was observed but counts mostly lower than preoperation were obtained in the following days. Before nephrectomy, the percentage of normoblasts varied from 14.4 to 41.9 (mean, 27.8) while 10 to 30 days after, it was 5.0 to 45.5 (mean, 19.2).

After bilateral nephrectomy. A rapid marrow depletion of normoblasts was observed in 15 dogs (Figure 1). Forty-eight hours after the second nephrectomy, the nucleated red cell count had fallen from a previous 5 to 45.5 per cent to 0 to 3.8 per cent. At this stage most of the basophilic normoblasts had disappeared and after 72 hours cells of the orthochromatic type with pyknotic nucleus were almost the only cells remaining. Subsequently, all normoblasts disappeared. Even when two dogs were severely bled daily after the second nephrectomy to a hematocrit of 10 and 12 per cent, a similar disappearance of normoblasts from the marrow was observed. Moderate bleeding of two dogs prior to the second nephrectomy elicited the same response (Figure 1).

After ureteral ligation. As shown in Figure 2, 72 hours after ligation the normoblasts had decreased from 16.6 to 38.5 to 4.4 to 21.7 per cent, but never completely disappeared. Nine and 10 days after ligation, normoblasts in two dogs were still 13.8 and 9.4, respectively. After one dog was bled daily after ureteral ligation to hematocrit 16 per cent, the normoblast even increased from 16.6 to 19.8 per cent the sixth day after ligation, despite a high urea level (280 mg. per 100 ml.).

3. *Reticulocytes*

In the normal dog 0 to 0.6 per cent reticulocytes were counted. Five days after bilateral nephrectomy, no reticulocytes were observed while sometimes 0.6 per cent reticulocytes were still present nine days after ureter ligation.

4. *Iron measurements*

The marrow picture was confirmed by the iron turnover rate and Fe^{59} utilization determinations (Tables I and II).

Plasma iron level. Plasma iron level of nephrectomized dogs, (81 to 267 μg. per 100 ml., average, 151) was higher than in normal (83 to 183 μg.

[1] This part of the work was performed at the Donner Laboratory, University of California, under contracts with the Atomic Energy Commission.

[2] We are greatly indebted to Dr. Donald C. Van Dyke who kindly provided us with the erythropoietic factor.

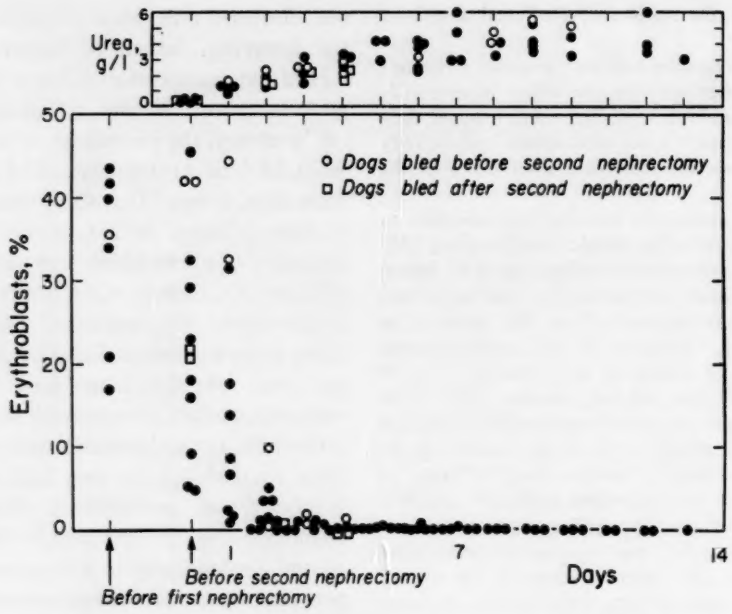

FIG. 1. DISAPPEARANCE OF ERYTHROBLASTS FROM THE MARROW AFTER BILATERAL NEPHRECTOMY

At the top of the graph are plotted the values for plasma urea before and after removal of the second kidney.

per 100 ml., average, 124) or in ureter-ligated dogs (58 to 136 μg. per 100 ml., average, 101).

Disappearance of Fe^{59} from plasma. Half-time plasma disappearance ($T_{1/2}$) of injected radioiron in bilaterally nephrectomized dogs was strikingly higher (201 to 435 minutes, mean, 299) than in normal dogs (39 to 153 minutes, mean, 89). After ureter ligation, normal values were obtained (58 to 171 minutes, mean, 96).

Iron turnover rate. Iron turnover was significantly lower in bilaterally nephrectomized dogs (0.09 to 0.49 mg. per Kg. per day, mean, 0.24) than in normal dogs where it averaged 0.64 mg. per Kg. per day (from 0.25 to 0.92 mg. per Kg. per day); $p < 0.01$. After ureter ligation iron turnover varied from 0.25 to 0.85 mg. per Kg. per day (mean, 0.48). The difference between nephrectomized and ureter-ligated dogs was significant ($p < 0.01$), but the difference between normal and ureter-ligated dogs was not significant.

Fe^{59} uptake in red cells. In normal dogs the maximal Fe^{59} incorporation varied from 58.7 to 80.9 (mean, 70.0). After bilateral nephrectomy Fe^{59} incorporation was practically abolished: from 0 to 12.5 per cent (mean, 5.3 per cent) (Table I). In Dog 2, despite important erythropoietic stimulation produced by bleedings before nephrectomy, Fe^{59} uptake (6.4 per cent) and iron turnover rate (0.20 mg. per Kg. per day) were also markedly reduced. In eight dogs with ureter ligation, incorporation in red cells three days after radioiron injection (that is, six days after the operation) averaged 46.8 per cent (16.3 to 76.9) as compared with 52 per cent (36 to 80.8 per cent) in normal controls. Five ureter-ligated dogs survived long enough to permit measurement of maximal uptake in red cells; this value averaged 61.3 per cent (35.8 to 80.8 per cent) (Table II). Only Dog 199 showed Fe^{59} red cell incorporation (35.8 per cent) lower than normal, although it was higher than nephrectomized values.

Storage of Fe^{59} in the liver. In two unilaterally nephrectomized dogs (No. 109 and No. 119) which had normal erythropoiesis, liver radioiron uptake was 16 and 24.6 per cent of the radioactivity injected. The same values were obtained in dogs with ligation of ureters (from 12.8 to 22.3 per cent, mean 16.1 per cent), whereas in bilater-

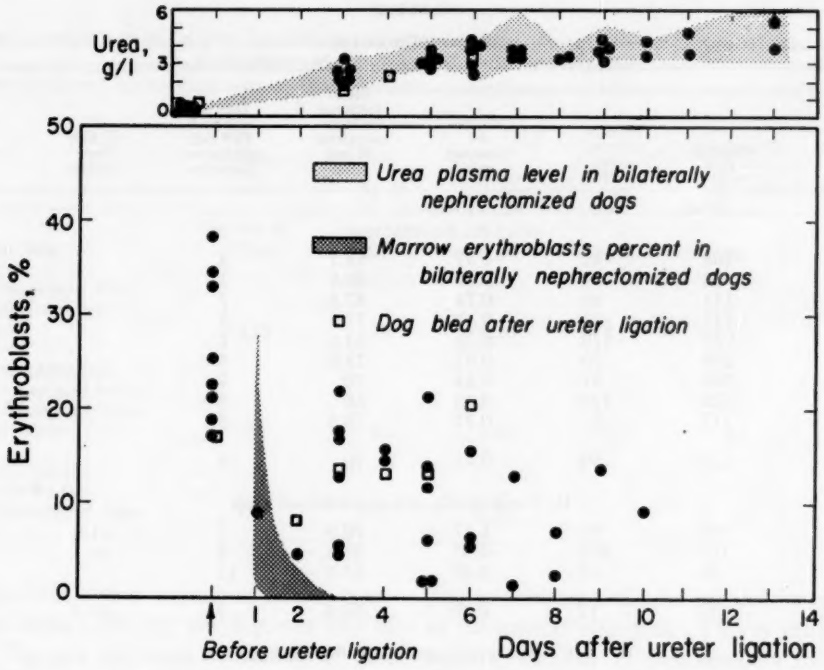

FIG. 2. ERYTHROBLAST COUNT IN THE MARROW BEFORE AND AFTER URETERAL LIGATION

At the top of the graph are plotted the values for urea plasma before and after ureteral ligation.

ally nephrectomized dogs in which erythropoiesis was suppressed, storage of Fe^{59} in the liver averaged 43.2 per cent (from 26.3 to 55 per cent).

Storage of Fe^{59} in the spleen. In unilaterally nephrectomized dogs as well as in ureter-ligated dogs, a very small amount of Fe^{59} was found in the spleen—from 0.5 to 2 per cent of radioactivity injected. The same magnitude of storage was obtained in bilaterally nephrectomized dogs except in one case (No. 11) which disclosed 13 per cent uptake in the spleen.

5. Effect of unilateral ureteral ligation followed by contralateral nephrectomy

One dog (No. 104, Table I) was submitted to ablation of the normal contralateral kidney 15 days after ureteral ligation. Erythropoiesis was similarly depressed as after bilateral nephrectomy: maximal Fe^{59} incorporation into red cells was 1.9 per cent, iron turnover was 0.27 mg. per Kg. per day and normoblast count was 1.2 per cent six days after nephrectomy.

6. Effect of erythropoietic factor on erythropoiesis of one dog before and after bilateral nephrectomy

Figure 3 shows the peak of reticulocytosis after two 150 mg. I.V. injections of human erythropoietic factor in a normal dog and the delayed increase of reticulocytes produced by the same amount of this active material after bilateral nephrectomy. The magnitude of the reticulocyte response after this operation cannot be interpreted because the dog died eight days after nephrectomy during the ascendent period of reticulocytosis. The same figure points out that the marrow was still relatively normal eight days after nephrectomy; at this time 9.5 per cent erythroblasts were counted compared with complete absence of erythroblasts observed in 15 bilaterally nephrectomized dogs after this delay (Figure 1). Plasma level of urea (312 mg. per 100 ml.) and potassium (7.5 mEq. per L.) was similar to other bilaterally nephrectomized dogs. Iron measurements are shown in Table III. Before nephrectomy, iron plasma level was unchanged before and

TABLE I

Iron measurement

Dog no.	Plasma Fe*	$T_{\frac{1}{2}}$	Plasma Fe turnover rate	Mean maximal Fe incorporation in red cells	Day of maximal Fe^{59} red cell incorporation	% Fe^{59} liver uptake	% Fe^{59} spleen uptake
	μg./100 ml.	*min.*	*mg./Kg./day*				
			A. Normal dogs				
A	108	82	0.52	58.7	8		
B	86	39	0.87	80.9	4		
C	111	66	0.74	67.5	7		
D	131	153	0.37	77.9	5		
E	83	120	0.25	63.2	7		
F	178	70	0.92	75.6	5		
G	183	91	0.84	70	7		
H	126	129	0.46	66	6		
I	112	62	0.77	70.5	5		
Mean	124	90	0.64	70	6		
			B. Unilaterally nephrectomized dogs				
109	167	66	1.17	70.5	5	24.6	1.5
119	117	102	0.57	57.8	4	16	0.5
196	81	47	0.59	83.4	11		
Mean	122	72	0.78	70.6	7	20.3	
			C. Bilaterally nephrectomized dogs				
6				2.9	5		
7	85	435	0.09	7.1	10	47.5	2
8	166	264	0.29	12.5	10	27.3	1.9
11†	81	201	0.20	6.4	8	49.5	13
17	211	245	0.34	0		55	1.3
18	267	241	0.49	0.6		51	0.9
19	106	285	0.21	1.3		32.6	0.6
27	125	375	0.12			40.7	0.6
28	170	348	0.19	11.8	4		
Mean	151	299	0.24	5.3	7.4	43.3	2.9
			D. Unilateral ureteral ligation followed by contralateral nephrectomy 15 days later				
104	128	229	0.27	1.9	5	23.4	0.4

* Iron plasma levels were measured on plasma samples taken 30 to 120 minutes after Fe^{59} injection.
† This dog was submitted to three 200 cc. bleedings the week before nephrectomy.

TABLE II

Iron measurements in ureter-ligated dogs

Dog no.	Plasma Fe	$T_{\frac{1}{2}}$	Plasma Fe turnover rate	% Fe incorporation after second day	% Fe incorporation after third day	Mean maximal Fe^{59} incorporation in red cells	Day of maximal Fe^{59} red cell incorporation	% Fe^{59} liver uptake	% Fe^{59} spleen uptake
	μg./100 ml.	*min.*	*mg./Kg./day*						
90	58	75	0.37		30.7	*		15.5	1
156	110	171	0.25			56	4	22.3	1.6
169	70	56	0.47	56.4	*			12.8	1.2
174	106	106	0.43			80.8	4	16.1	1.7
185	90	89	0.44		41.4	*		13	0.8
193	131	64	0.85			71.8	5	16	1.6
198	108	110	0.45			62	4	15.7	0.5
199	136	99	0.55			35.8	5	17.6	0.8
Mean	101	96	0.48			61.3	4.5	16.1	1.2

* Dog died.

TABLE III

Iron measurements in one dog in the normal state, after injection of erythropoietic factor and after bilateral nephrectomy plus injection of erythropoietic factor

	Plasma Fe level	$T_{\frac{1}{2}}$	Fe turnover	% Fe uptake in red cells after 2 days	Maximal % Fe^{59} uptake in red cells	Day of maximal Fe^{59} red cell uptake
	μg./100 ml.	*min.*	*mg./Kg./day*			
Normal dog	126	129	0.46	29	64	6
After injection 300 mg. erythropoietic factor	122	48	1.28	83	101	5
After bilateral nephrectomy and injection 300 mg. erythropoietic factor	35	44	0.40	20	83	5
Average values of 9 bilaterally nephrectomized dogs.	151	299	0.24		5.3	7.4

after erythropoietic factor injection. Iron turnover increased from 0.48 mg. per Kg. per day to 1.28 mg. per Kg. per day, and maximal Fe^{59} red cell uptake, which was 64 per cent before, reached 101 per cent after injection. Furthermore, the speed of the Fe^{59} red cell uptake was strikingly increased; 24 hours after Fe^{59} injection, 32 per cent of the radioactivity appeared in the red cells, while only 13.5 per cent was counted after this delay in the normal dog without administration of the erythropoietic factor.

Injection of the erythropoietic factor in the dog after bilateral nephrectomy is associated with a fall in plasma iron to 35 μg. per 100 ml. Iron turnover was of the same magnitude as in the normal dog without erythropoietic factor injection (0.40 mg. per Kg. per day) and maximal Fe^{59} red cell uptake averaged 83 per cent. Strictly speaking, this last result is not comparable with the previous red cell uptake because of reduction of the iron labile pool.

DISCUSSION

The data presented indicate that bilateral nephrectomy of the dog suppresses erythropoiesis. After bilateral nephrectomy normoblasts disappeared from the marrow. The initial disappearance of basophilic and polychromatic forms suggests normal erythron maturation, but arrest of stem cell differentiation into normoblasts. Removal of both kidneys abolishes the capacity of the dog to produce red cells regardless of the severity of the anemic stimulus. This is illustrated by the aplastic marrow of two severely bled nephrectomized dogs. In anemic anuric patients, a similar marrow picture is observed (6, 13). It must be emphasized that disappearance of normoblasts from the marrow after bilateral nephrectomy was not observed by other investigators working on the rabbit (8, 27). Since the normoblast count is

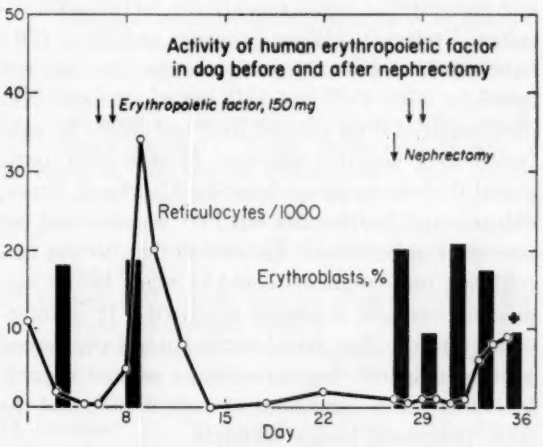

FIG. 3. EFFECT OF HUMAN URINARY ERYTHROPOIETIC FACTOR ON RETICULOCYTES AND BONE MARROW ERYTHROBLASTS IN A DOG BEFORE AND AFTER BILATERAL NEPHRECTOMY [3]

[3] From "Kinetics of Cellular Proliferation," F. Stohlman, Jr., Ed. New York, Grune and Stratton, 1959.

a relative one, the striking difference between the dog and rabbit could be explained by a greater degree of granulopoiesis after nephrectomy in the dog. However, the complete disappearance of normoblasts and the fact that the basophilic and polychromatic forms disappear even sooner indicate that this phenomenon is not due to dilution by granulopoiesis. This dilution mechanism, however, could explain the transient reduction in normoblasts after unilateral nephrectomy where basophilic and polychromatic normoblasts are still present. After ureteral ligation, these forms are also maintained and reduction of nucleated red cells could also be explained by increased leukopoiesis. However, the lowered normoblast count after ligation might also be secondary to progressive renal damage involving reduced erythropoietic secretion. It is shown (Dog no. 104, Table I) that if one ureter is ligated and the opposite normal kidney removed 15 days later, the same picture is obtained as after bilateral nephrectomy. Finally, some toxic effect of waste products on marrow could not be excluded.

Ferrokinetic results agree with morphologic observations of the bone marrow. The observed low iron turnover, the low Fe^{59} red cell incorporation, the increased Fe^{59} storage in the liver after bilateral nephrectomy are all characteristic of marrow hypoplasia. Neither surgical wound infection nor malnutrition could explain the ferrokinetic results. Freireich, Miller, Emerson and Ross (28) have shown that local infection in the dog does not interfere with Fe^{59} red cell incorporation. Nephrectomized dogs refused food and might be subjected to a negative nitrogen balance after peritoneal dialysis as pointed out by Muirhead, Jones, Stirman and Lesch (29) but Fe^{59} was injected before start of peritoneal dialysis so that certain ferrokinetic measurements could be made before significant nitrogen depletion occurred. It is interesting to note that ferrokinetic studies performed on ureter-ligated dogs are almost normal regardless of the same conditions of malnutrition and the same peritoneal lavage schedule.

The small discrepancy in the labile iron pool between normal and bilaterally nephrectomized dogs cannot explain the low Fe^{59} uptake. Furthermore, it can be seen that even nephrectomized dogs (Nos. 11 and 19, Table I) with normal plasma iron levels, have a low iron uptake (6.4 and 1.3 per cent). Fe^{59} measurements of the spleen showed that there was no red cell sequestration. Only Dog 11 which had been bled before investigation disclosed spleen Fe^{59} uptake of some importance. Only about 50 per cent of the radioactivity injected in the nephrectomized dog can be accounted for in the red cells, liver and spleen. Almost all radioactivity disappears from the plasma within 24 hours and only insignificant amounts of Fe^{59} are detected with irrigating fluid. It seems not unlikely that some Fe^{59} might be located in marrow reticuloendothelial system.

Retention of toxic products due to absence of renal secretory function is obviously not the cause of arrested erythropoiesis after bilateral nephrectomy. Dogs with ureteral ligation maintained erythropoietic function despite the same state of azotemia and hyperkalemia. Of particular interest is the observation that after administration of erythropoietic factor a bilaterally nephrectomized uremic dog can maintain a normal erythroblastic marrow picture. It seems thus that the bone marrow is able to respond to erythropoietic hormone in spite of uremia or absence of the kidneys. These results strongly suggest that the kidney produces an erythropoietic factor. In the rat, Jacobson and associates (16) and Goldwasser, Fried and Jacobson (30) have pointed out the lack of erythropoietic factor response to hypoxia after nephrectomy, but other workers did not obtain the same results (31). On the other hand, Erslev (27), working on the rabbit, concluded that the kidney did not play a role in erythropoietic function. Other investigations point to the role of the kidney in erythropoietic factor production. Van Lessen, Stefanini and Smith (32) have shown in the dog that partial ligation of only one renal vein induced polycythemia. These findings could explain the association of renal carcinoma and polycythemia observed in some patients (33–35). More recently Osnes (36) observed in mice that irradiation of both kidneys reduced the erythropoietic factor responses which normally follow bleedings. Recent demonstration of rapid disappearance of the erythropoietic factor from plasma of anemic dogs after bilateral nephrectomy added strong evidence to support the role of the kidney in erythropoietic factor production (37). The regulatory role of the kidney in erythropoiésis could be linked to the very specialized renal circulation.

It is known that the oxygen saturation of blood in the renal vein is very high (averaging 80 to 90 per cent) and that normal renal arteriovenous oxygen difference is about 2.4 ml. per 100 ml. blood (38, 39) as compared to the mixed venous and arterial blood oxygen difference of 3.4 to 6.1 ml. per 100 ml. blood. According to the "cell separation" theory of Pappenheimer and Kinter (40), "The peritubular circulation is supplied with a cell-poor component of blood and the oxygen saturation in the blood leaving the actively metabolizing tissue of the kidney may be far lower than that in the renal vein." Thus normally most of the tubules should be at the borderline of hypoxia. Such a system could be very sensitive to blood oxygenation if, when the arterial hematocrit decreases, cell separation might increase, making the tubules more hypoxic. It could be possible that some oxygen-sensitive tubular endocrine system [juxtaglomerular apparatus, for instance, as suggested by Osnes (36)] might respond to a lack of oxygen by increased erythropoietic factor production.

SUMMARY

The relationship between the kidney and erythropoiesis was investigated. Forty-four mongrel dogs were used of which 15 dogs were subjected to bilateral nephrectomy and nine to ureter ligation. Controls consisted of nine normal dogs and three unilaterally nephrectomized dogs. Uremic dogs were maintained for 7 to 15 days by peritoneal lavage.

Removal of both kidneys was followed by a rapid depletion of erythroblasts from the marrow; often none could be found after 72 hours. Iron measurements showed a low iron turnover and reduced Fe^{59} incorporation in red cells of nephrectomized dogs.

Although bilateral ureteral ligation resulted in the same elevation of blood urea nitrogen and the same degree of starvation as in the nephrectomized group, erythropoiesis was essentially normal. The erythroblast population of the marrow was maintained. Although the plasma iron turnover was reduced, it was significantly higher than in the nephrectomized group. Fe^{59} incorporation into red cells was of the same magnitude as in the control group.

Maintenance of marrow erythroblasts was observed after injection of erythropoietic factor into one bilaterally nephrectomized dog.

It is concluded from these data that the cessation of erythropoiesis after nephrectomy cannot be accounted for by urea intoxication and that the kidney is probably the source of an erythropoietic factor.

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SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS IN FRACTIONS OF PERIPHERAL NERVE * †

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Embryological and histological investigations have indicated that both axons and Schwann cells are necessary in the growth and maintenance of the myelin sheaths (2–4). Ben Geren (5) found that the myelin lamellae are formed as a result of winding around the axon layer by layer, the ends remaining attached on the inside to the axolemma and on the outside to the Schwann cell membrane. The Schwann cells have also been observed to deposit a fibrous layer binding them tightly to the nerve axons before the onset of myelination (4). The remainder of the nerve connective tissue is produced from fibroblasts (6).

The chemical composition of the lipid components of the myelin layers has been studied in some detail (7, 8), and the principal components are thought to be free cholesterol, cerebroside, sphingomyelin and phosphatidyl serine with a molecular ratio of 2 cholesterol: 2 phospholipid: 1 cerebroside. The biochemical processes involved in myelinization have been shown to include synthesis of cholesterol and phospholipids (9). The site of production of these lipids is uncertain. The fact that myelinization does not take place until Nissl substance maturation has occurred indicates that myelin formation depends on axon development. When myelinization takes place, it occurs simultaneously in several different areas. These are the areas in which Schwann cells are in contact with the nerve (4).

It was the purpose of this investigation to explore the relative roles of axons versus connective tissue elements and Schwann cells in the synthesis of cholesterol and fatty acids in adult peripheral nerve *in vitro*.

METHODS

Fractionation. Sciatic nerve segments 3 to 4 mm. long were removed from cats, some saved as controls and the others gently homogenized with 2 cc. Krebs-Ringer bicarbonate buffer in Dounce homogenizers with loose fitting plungers. This and the following procedures were carried out at 4° C. Two fractions were obtained: a milky homogenate, and an insoluble residue called the sheath fraction. These fractions were separated by simple decantation of the homogenate. The homogenate was centrifuged at 800 G for 10 minutes.

Incubation. Control nerves were placed in one flask modified according to Baruch and Chaikoff (10), the homogenate in another, and into a third the sheath was placed. The sheath and homogenate were recombined and placed in a fourth flask. The substrates used were added in the following concentrations: glucose, 9×10^{-3} M; potassium acetate, 1×10^{-3} M; and acetate-1-C^{14}, 8×10^{-3} M, in a total volume of 2.45 ml. The flasks were gassed with 95 per cent oxygen and 5 per cent carbon dioxide and stoppered with serum caps. The flasks were incubated in a Dubnoff shaker for two hours at 37° C.

ANALYTICAL METHODS

Deoxyribonucleic acid (DNA) analysis. The composition of the two fractions was studied with analysis of DNA and microscopic smears. Extraction of the nerves was based on Ceriotti's modification (11) of Ogur and Rosen's technique (12). After two extractions with absolute alcohol and two with ether, acid soluble components were removed with 2 per cent perchloric acid. The nucleic acids were then extracted from the residue with hot 10 per cent perchloric acid. The DNA in the perchloric extract was determined with Ceriotti's indole method (13). Spleen DNA was used for standard solution. All determinations were carried out in duplicate and the results averaged.

$C^{14}O_2$. At the end of the incubation, the flasks were placed in an ice bath. One ml. of alkaline Hyamine® 10X [1] in methanol-toluene 1:1, prepared as described by Passman, Radin and Cooper, (14) was injected through the serum cap into the center well, and the carbon dioxide was collected as previously described (15). The Hyamine®-$C^{14}O_2$ solution was next transferred quantitatively

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[1] Hyamine® 10X is the trade name for *p*-diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate (The Rohm and Haas Company).

with toluene into a 10 ml. volumetric flask. A 2 ml. aliquot was placed in 5 ml. of a 0.4 per cent solution of diphenyloxazole in toluene, and the C^{14} assayed in a Packard Liquid Scintillation Counter.

Cholesterol-C^{14} determination. The nerve and incubation mixture was saponified by adding 1 ml. of 90 per cent (weight per volume) potassium hydroxide and heating in an autoclave at 15 pounds' pressure in 120°C. for one hour (15). The saponified mixture was then transferred into a 250 ml. Erlenmeyer flask and ethanol was added to give a 50 per cent solution. The nonsaponifiable material was next extracted with petroleum ether, and the cholesterol fraction was isolated as the digitonide by the procedure of Sperry and Webb (16). This fraction also contains other 3β-sterols, which in the following are included under the term cholesterol. The digitonide was dissolved with heating in 2.2 ml. of methanol and 1 ml. of the solution was added to 16 ml. of the liquid scintillation solution for C^{14} determination.

Fatty acid-C^{14} determination. After removing the nonsaponifiable material as described above, the 250 ml. Erlenmeyer flask was heated on a steam bath to remove the ethanol. Water was then added to give a volume of about 5 to 6 ml. and the mixture was acidified below pH 3.0 with concentrated sulfuric acid and chilled. The fatty acids were then extracted into a measured volume of petroleum ether by vigorous shaking for 15 minutes (15) at room temperature. An aliquot was washed with water and assayed for C^{14}.

Calculations. All determinations were carried out in duplicate. Because the values obtained agreed within 10 per cent, they were averaged and given in the tables as averages. Carbon dioxide-C^{14} samples counted from 1,770 to 133,115 cpm. Fatty acid samples contained 900 to 24,660 cpm. The cholesterol fractions had from 5,880 cpm to the same level as background. The background counts varied from 6.5 to 14.7 cpm. All aliquots with counts lower than 100 cpm were counted for 10 minutes, samples between 100 and 200 cpm for 5 minutes. Values over 200 cpm were averaged from four one minute counts. Background was always counted in comparable fashion and checked against every fourth sample of the same counting time. The maximal 0.95 counting error in carbon dioxide and fatty acid analysis is ± 0.4 mμMole. In cholesterol synthesis a 0.95 counting error of 33 per cent appears in samples with counts lower than 36 cpm and these are therefore listed as lower than 1 mμMole × 10^{-2}.

TABLE I

Deoxyribonucleic acid (DNA) in cat nerve homogenate and residue

Expt. no.	μg. DNA/100 mg. homogenate	μg. DNA/100 mg. residue (sheath fraction)
1	<5	68
2	10	41
3	<5	86
4	<5	54
5	6	59
6	<5	82
7	8	64
8	<5	78

RESULTS

DNA analysis

The results of the DNA analysis are seen in Table I. Eight nerves were analyzed. The amounts of DNA found in the homogenate fractions from five nerves was less than 5 μg. per 100 mg. substrate. This is the lower limit for the analysis method. In the other three, small amounts of DNA were detected. Microscopic analysis of the homogenate showed fragments of nerve fibers with or without myelin sheath. A few Schwann cells could also be seen.

In contrast, the residue fraction contained remnants of the endo- and perineurium, Schwann cells and some nerve axon bundles. The DNA contents varied from 41 to 86 μg. DNA per 100 mg. with residue. These amounts approximate the figure given by Logan, Mannell and Rossiter (17) for total nerve, *i.e.*, 60 μg. DNA per 100 mg. wet nerve tissue.

The results of the DNA analysis indicated that little nuclear material was left in the homogenate fraction. This fraction consequently contained mostly nerve axons and myelin. The residue fraction included the majority of the Schwann cells as well as the connective tissue from the peripheral nerve.

TABLE II

Incorporation of acetate-1-C^{14} into cholesterol in millimicromoles × 10^{-2}. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	11	61	2	7	6	130	49	36
Homogenate	2	2	<1	<1	<1	<1	<1	1
Sheath	2	9	6	6	10	6	2	1
Homogenate and sheath	3	3	<1	7	34	2	4	1

Incorporation of radioactive acetate

Cholesterol synthesis from acetate-1-C^{14} in the unhomogenized controlled nerve ranged from 2 to 130 mμMoles × 10^{-2} (Table II). After separation into fractions, the sheath fraction retained the

ability to synthesize cholesterol whereas five out of eight homogenate fractions incorporated only a negligible amount of radioacetate into cholesterol. Recombination of the two fractions did not appreciably increase cholesterol synthesis (with the exception of Experiment 5).

The synthesis of fatty acids differed in two aspects from that of cholesterol. First, the amounts synthesized in the homogenate were larger than those obtained from the sheath fraction in six out of eight experiments (Table III). Second, the synthesis in the homogenate was higher than that in the control nerve in half of the experiments. Again, recombination of the two fractions seemed to have no stimulatory action on the fatty acid synthesis.

Production of carbon dioxide-C^{14} varied in the control nerve from 16 to 153 mμMoles (Table IV). After homogenization the homogenate fraction evolved smaller amounts of radioactive carbon dioxide (3 to 51 mμMoles) than the sheath fraction (6 to 219 mμMoles).

TABLE III

Incorporation of acetate-1-C^{14} into fatty acids in millimicromoles. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	5.4	14.0	16.5	1.9	46.7	4.2	10.0	11.1
Homogenate	2.4	17.8	6.7	9.2	52.8	3.8	3.7	19.9
Sheath	2.7	10.9	1.9	6.5	33.8	2.1	7.6	8.6
Homogenate and sheath	4.3	8.7	5.9	6.6	11.5	32.6	3.4	9.4

TABLE IV

Acetate-1-C^{14} to carbon dioxide in millimicromoles. Cat sciatic nerve

Expt. no.	1	2	3	4	5	6	7	8
Control (unhomogenized)	70	45	102	63	153	72	43	16
Homogenate	3	4	3	4	51	36	4	7
Sheath	10	16	34	34	90	219	6	9
Homogenate and sheath	18	13	20	66	226	90	5	6

DISCUSSION

The homogenate fraction obtained with our technique contained axon and myelin fragments as well as soluble components from disrupted Schwann cells and connective tissue. The sheath fraction included the majority of the Schwann cells and the connective tissue of the peripheral nerve. In the axon fractions, fatty acids were synthesized but little or no cholesterol was formed; in the sheath fractions, definite amounts of cholesterol were synthesized and, in addition, some fatty acids.

It would appear that cholesterol synthesis from acetate could not take place in axon and myelin fragments *in vitro*. Neither did the necessary enzyme systems seem to be soluble enough to be extracted from the total nerve, with our technique. It has been stated that cholesterol cannot be synthesized by adult brain tissue (18). If we assume that the immature nerve tissue can synthesize cholesterol, then one or more of the enzyme systems used disappears. The possibility also remains that the nerve cell nucleus is necessary for this synthesis. It seems more likely that in the peripheral nerve, cholesterol synthesis from acetate takes place in the Schwann cells or fibroblasts which surround the nerve axon. It has been shown that fibroblasts in culture can produce cholesterol (19). In view of the previously stated morphological evidence that Schwann cells participate in myelination, it seems more attractive to assume that they supply the cholesterol necessary for the myelin sheath. Additional evidence to support their role in lipid synthesis has been brought out by the demonstration of an increase of Schwann cells in response to injury (20), an increase which parallels the increase in cholesterol synthesis (21). Furthermore, if the amount of C^{14} incorporated into lipid by a single Schwann cell is calculated (22), the greatest activity occurs at peak level of myelinization or about 20 days after birth.

Fatty acid synthesis must play a major role in the maintenance of the myelin sheath. The synthesis of sphingomyelin and glycerol phospholipid requires fatty acids of probably different chain lengths. But, also, the axon lipids contain fatty acids. It is therefore not remarkable that fatty acid synthesis would take place in the axon fraction. The homogenization procedure used does not exclude the possibility that soluble cofactors

from the sheath fraction pass over to the homogenate. The additional increase of such cofactors as would occur on recombination of the two fractions does not seem to stimulate fatty acid synthesis any further. However, maximal stimulation may have already occurred. The fact that the homogenized nerve occasionally incorporates more acetate-1-C^{14} into fatty acid than does the intact nerve could be explained on the basis of a higher penetration of substrate into the axon fragment. On the other hand, occasionally lower rates of synthesis of fatty acids are observed in the homogenate; this could be explained on the basis of liberation of nucleotidases during the homogenization procedure. It has been shown that triphosphopyridine nucleotide (TPN) is rate limiting in fatty acid synthesis in liver (23). We have found that fatty acid and cholesterol synthesis in normal rat nerve is stimulated by addition of the reduced triphosphopyridine nucleotide (TPNH) generating system (24). An excessive destruction of TPN may therefore limit lipogenesis in the axon. Because the sheath fraction could never be completely freed of axon tissue, the question of how much fatty acid synthesis normally takes place in the Schwann cell could not be answered.

The CO_2 production does not seem related to either the amounts of cholesterol or fatty acids produced. Neither does there seem to be any competition between oxidative processes and lipogenesis. The higher carbon dioxide synthesis in the sheath fraction is thought to reflect simply the larger amount of cellular material.

The findings stress the fact often pointed out by histologists that myelinization is dependent on the presence of both sheath elements and nerve fibers. According to Speidel (3), a nerve sprout coming from a myelinated nerve differs from a nonmyelin emergent fiber. The former in combination with a primitive Schwann cell leads to the formation of a new myelinated segment; the latter ordinarily does not. This would indicate that there is a basic difference in the structure of myelinated and nonmyelinated nerve fibers which influences the degree of myelination. The presence of Schwann cells around both types of nerves could be taken to indicate that the limiting factor in myelinization may be the quantity of specific fatty acids supplied by the axon.

SUMMARY

Gentle homogenization of cat sciatic nerves resulted in the separation of two fractions. One is a homogenate containing chiefly axons and myelin, the other is a sheath fraction, containing fibrous tissue and Schwann cell nuclei.

In vitro cholesterol synthesis from acetate occurs almost exclusively in the sheath fraction.

In vitro synthesis of fatty acids often is more active in the homogenate fraction.

The possibility is discussed that such a separation in synthetic properties may reflect the relative role of the axons and Schwann cells in peripheral myelinization.

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CARBOHYDRATE METABOLISM IN PREGNANCY. I. THE METABOLISM OF INSULIN BY HUMAN PLACENTAL TISSUE * †

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Much clinical evidence would suggest that there is a change in carbohydrate metabolism during pregnancy (2–8). In the past, the diabetogenic effects of pregnancy have been attributed to an increased elaboration of hormonal contrainsulin factors. An alteration in the degradation of insulin during pregnancy might constitute a supplemental or alternative mechanism. It has been shown by Mirsky, Perisutti and Dixon (9, 10) and Tomizawa, Nutley, Narahara and Williams (11, 12) that various animal tissues, such as liver, contain relatively specific enzymes for the proteolytic inactivation of insulin. To date, such systems have not been studied in isolated preparations of human tissues. In the present study, the presence of an active, enzymatic mechanism for the proteolytic degradation of insulin has been demonstrated in human placental tissues. The characteristics of the system have been defined and its potential contributions to maternal insulin economy have been evaluated.

MATERIALS

1. I^{131}-insulin. Crystalline beef insulin (Lilly zinc insulin No. 535664 assaying 27 units per mg.) was purchased from Abbott Laboratories, Oak Ridge, Tennessee, in solutions containing 0.1 mg. insulin per ml. It had been labeled with I^{131} to an initial specific activity of 4 to 7 mc. per mg. The iodination entailed the substitution "approximately one atom of iodine per molecule of insulin, assuming a molecular weight of 6,000 for the insulin" (13). Upon receipt in this laboratory, the I^{131}-insulin was immediately diluted with four parts of phosphate-buffered sodium chloride (116 mMoles per L.) containing 5 per cent human serum albumin (HSA). Thereafter, the material was dialyzed at 4° C. for 24 hours against serial changes of sodium chloride (135 mMoles per L.) buffered at pH 7.4 with sodium phosphate (10 mMoles per L.). The resultant dialyzed and buffered albumin-insulin mixtures were employed as stock solutions. No stock solution was employed for more than 10 days. Between experiments, stock solutions were stored in the frozen state.

More than 98 per cent of the total radioactivity in the stock I^{131}-insulin solutions could be precipitated with trichloroacetic acid (TCA). Various preparations were analyzed for homogeneity by hydrodynamic flow paper chromatography (14) and by paper electrophoresis on Whatman No. 3 filter paper, in barbituric acid-sodium barbiturate buffer, pH 8.6, $r/2$ 0.05. As judged by the latter criteria, products of radiation damage or spontaneous degradation did not exceed 7 per cent of the total radioactivity. When three separate stock solutions were diluted to 1×10^{-4} units per ml., their hypoglycemic potency in the rat epididymal fat pad assay system (15) was not significantly different from that of comparable quantities of unlabeled crystalline insulin.

For individual experiments, stock solutions of I^{131}-insulin were supplemented with unlabeled "glucagon-free" crystalline zinc insulin.[1] The unlabeled insulin had been dissolved in water with the addition of a few drops of 0.01 N HCl and solutions of 2 mg. insulin per ml. were maintained at pH 3.9 during storage at 4° C.

2. Placentas. No tissues from patients with obstetrical complications or disturbances of carbohydrate metabolism were included in the present study. Term placentas were obtained following vaginal delivery or elective repeat Caesarian section. Retroplacental blood was expressed manually. Immediately thereafter, placental segments were excised with blunt dissection scissors, freed from adherent membranes, briefly blotted on filter paper

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[1] Crystalline zinc insulin (No. 499667) assaying 25 units per mg. and crystalline glucagon (No. 258-235-B-54-2) were generously provided by Dr. O. K. Behrens and Dr. C. W. Pettinga of Eli Lilly Co. Somar-A (Bovine Growth Hormone), Armour No. R50109, and Panlitar (Ovine Lactogenic Hormone), Armour No. R10109, were gifts of the Endocrinology Study Section, National Institutes of Health. Thytropar (Bovine Thyrotropin Armour No. R2402), Bovine Corticotropin ACTH (Armour No. R68406C) and crystalline bovine albumin (Armour), were obtained commercially.

and frozen on solid carbon dioxide. All manipulations were completed within 5 minutes following delivery of the placenta. Weights of the placentas and membranes were measured with a single pan balance, accurate to ± 1 Gm. For experiments involving placental slices, the tissues were not frozen but rather wrapped in cellophane and transferred to the laboratory on cracked ice in Dewar flasks.

Obstetrical management of the patients consisted of routine premedication with 90 to 120 mg. Seconal® and 0.4 mg. atropine. Caesarian sections were performed with spinal anesthesia. In some instances 1 ml. (10 I.U.) Pitocin® was administered intramuscularly prior to expulsion of the placenta. Placental enzymatic activity did not appear to be significantly affected by these medications. Comparable results were observed in two experiments in which placentas were obtained following precipitous delivery before any drugs could be administered.

METHODS

1. Slice experiments. Tissue slices of 0.5 mm. thickness were prepared by free hand slicing, floated on chilled saline, and washed in three portions of saline to remove adherent blood. Two hundred ± 10 mg. slices were introduced into open 20 ml. beakers containing 2.0 ml. of modified Krebs-Ringer phosphate enriched with 2 mg. per ml. of glucose (KRP-Glucose). Following temperature equilibrations, 0.2 ml. of insulin solutions was introduced and incubation was performed in the Dubnoff apparatus in an atmosphere of 100 per cent oxygen.

2. Homogenate experiments. Weighed frozen aliquots of placenta were homogenized in Potter-Elvehjem ground-glass homogenizers with two parts 0.1 M, pH 7.4, phosphate buffer to yield 1:3 homogenates. For the *standard experimental procedure,* the homogenates were centrifuged for 30 minutes in an International refrigerated centrifuge at 15,000 × G and 4° C. One-half ml. aliquots of the supernatant solutions were added to 1 ml. of 0.1 M, pH 7.4, phosphate buffer contained in open 20 ml. reaction vessels. Insulin solutions were prepared by combining diluted stock solutions of I^{131}-insulin with varying quantities of unlabeled insulin in 0.1 M, pH 7.4, sodium phosphate buffer containing 25 mg. of HSA per ml. Enzyme and substrate preparations were equilibrated at 38° C. Thereafter, 0.5 ml. quantities of the insulin solutions were introduced into the reaction vessels and incubation was performed at 38° C. in the Dubnoff apparatus (shaking rate, 100 cycles per min.). Thus, the final reaction mixture in the *standard experimental procedure* consisted of 2 ml. of pH 7.4 phosphate buffer containing 12.5 mg. of HSA, varying amounts of insulin, and the 15,000 × G soluble supernatant solution derived from 166 mg. of wet weight placenta. Control studies had indicated that the 6.2 mg. HSA per ml. did not alter enzymatic activity although it prevented adsorption of insulin to glassware and the spontaneous degradation of dilute iodoinsulin (*vide infra*). Moreover, this amount of HSA provided sufficient carrier protein for precipitation with TCA. In all studies, concurrent incubations were performed with blank reaction vessels containing only buffer and the insulin-HSA mixtures.

3. Assay of insulin degradation. Specific techniques employed to characterize the products of insulin degradation will be discussed separately in the Results section. In the *standard experimental procedure,* assay was based on the solubility characteristics of the radioactivity in TCA. At timed intervals throughout incubation, 0.2 ml. aliquots of reaction mixture were removed and introduced into 13 × 100 mm. counting tubes containing 2 ml. of 20 per cent TCA. Radioactivity was partitioned into TCA-soluble and TCA-insoluble fractions by centrifugation. The insoluble residues were washed by resuspension in 2 ml. of 20 per cent TCA and centrifuged. The TCA supernatant fractions were pooled and the washed insoluble moieties were dissolved in 4 ml. of 30 per cent KOH. Radioactive assay of the TCA-soluble and TCA-insoluble fractions was performed in a Nancy Wood well-type scintillation counter with a sensitivity of 1.0×10^6 cpm per μc. I^{131} and background of 125 cpm. Sufficient counts were observed to reduce the probable error of the measurement to less than ± 3 per cent. After appropriate corrections for blanks, the TCA-soluble radioactivity in individual specimens (*i.e.,* nonprecipitable I^{131}) was expressed as a percentage of the total radioactivity within the system (*i.e.,* the sum of the TCA-soluble plus TCA-insoluble radioactivity).

In the slice experiments, 2 ml. of 20 per cent TCA was added to the vessels at the end of incubation. The flask contents were quantitatively transferred to Potter-Elvehjem ground-glass homogenizers and were homogenized preparatory to fractionation into TCA-soluble and TCA-insoluble components as above.

4. Analytical techniques. Paper electrophoresis was performed with the apparatus and techniques described elsewhere (16). Butanol-acetic acid-water 78:10:12 (B Ac) and butanol-dioxane-2 N NH_3 4:1:5 (BDA) were employed for chromatography. Herein, portions of untreated reaction mixture, or TCA-soluble supernatant were applied to Whatman No. 1 filter paper after being supplemented with carrier L-thyroxine (T_4), L-triiodothyronine (T_3), L-monoiodotyrosine (MIT), L-diiodotyrosine (DIT), sodium iodide, and 1×10^{-3} M 1-methyl-2 mercaptoimidazole. The carrier compounds were localized by successively spraying with solutions of 0.1 per cent palladium chloride and diazotized sulfanilic acid. Duplicate chromatograms were also sprayed with 0.1 per cent ninhydrin in acetone. Distribution of radioactivity in individual electrophoretograms and chromatograms was qualitatively localized by radioautography and quantified with the use of an electronically integrating, automatically recording, strip-scanner developed in this laboratory (17). A micro-Kjeldahl method was employed for analysis of total nitrogen in duplicate aliquots of placental extracts. Blood glucose was measured by the method of Somogyi as modified by Nelson (18).

RESULTS

1. Methods

The published findings of others (19–22) prompted a re-examination of the potential methodological artifacts in the use of I^{131}-insulin in *in vitro* systems. Representative experiments are described in detail below.

A. Adsorption to glassware. Ten uncovered 20 ml. beakers were filled with constant tracer quantities of I^{131}-insulin, varying amounts of crystalline unlabeled insulin, and sufficient 0.1 M, pH 7.4, phosphate buffer to yield 2.0 ml. systems containing 0.25 to 500.25 μg. insulin per ml. (Table I). The vessels were counted by being placed on a fixed aluminum disk directly above a scintillation detector head. Thereafter, they were incubated for 30 minutes at 38° C. in the Dubnoff apparatus. Following incubation, the beakers were emptied into separate 10 ml. volumetric flasks containing 50 mg. HSA and refilled with 2.0 ml. of phosphate buffer. Repeat radioactive assay was performed to assess the "percentage of the initial radioactivity" which had been retained within each beaker ("glassware adsorption"). This exceeded 25 per cent of the initial counts in the vessels which originally did not contain more than 2.5 μg. insulin per ml. (Column A, Table I). The ease with which the "adsorbed" radioactivity could be removed by washing was assessed as follows: beakers were decanted into the same volumetric flasks; a single washing with 3.0 ml. of phosphate buffer was performed; then the beakers were counted for a third time. As shown in Column B, Table I, more than 5 per cent of the initial radioactivity still remained in these beakers.

TABLE I

Adsorption of insulin to glassware and spontaneous degradation of insulin during incubation in aqueous media

Reaction mixture: 2 ml. pH 7.4 phosphate buffer containing 0.25 to 500.25 μg. insulin per ml. and no other protein. Incubation: 30 minutes, 38° C.

No.	Insulin	HSA	A*	B†	C‡
	μg./ml.	mg./ml.	%	%	%
1	0.25	0	46.7	19.6	8.9
2	0.75	0	48.7	13.8	7.9
3	1.50	0	32.9	7.7	6.0
4	2.50	0	27.6	6.4	4.4
5	5.00	0	15.3	3.8	3.9
6	10.25	0	8.5	2.3	1.2
7	50.25	0	4.8	1.4	0.7
8	100.25	0	3.5	0.7	0
9	200.25	0	3.1	0.5	0
10	500.25	0	3.9	0.6	0

* A: "per cent of initial radioactivity" retained in beaker after decanting the original 2.0 ml. suspending medium (*i.e.*, "glassware adsorption").

† B: "per cent of initial radioactivity" retained in beaker after washing successively with 2.0 ml. and 3.0 ml. of phosphate buffer.

‡ C: minimal estimate of "per cent of initial radioactivity" rendered soluble in TCA during incubation (*i.e.*, "spontaneous degradation").

B. Spontaneous degradation. The albumin-containing 10 ml. volumetric flasks into which the insulin and the beaker washings had been decanted in the above experiments were employed to assess spontaneous degradation. Control volumetric flasks were prepared by directly combining 0.50 μg. I^{131}-insulin with 50 mg. HSA without preliminary incubation. Flasks were diluted to constant volume and duplicate 1.0 ml. aliquots from each were fractionated into TCA-soluble and TCA-insoluble components. After appropriate correction for TCA-soluble radioactivity in control flasks, minimal estimate of spontaneous degradation during incubation was obtained by multiplying *a*) the percentage of the initial radioactivity which had been transferred to the volumetric flasks (*i.e.*, 100 per cent minus Column B, Table I) by *b*) the percentage of the radioactivity in the volumetric flasks which was soluble in TCA. Results are listed in Column C, Table I. During aerobic incubation of aqueous systems containing less than 2.5 μg. insulin per ml., more than 5 per cent of the insulin is rendered soluble in TCA.

Similar values for glassware adsorption and spontaneous degradation were obtained in repeat studies with varying proportions of I^{131}-labeled and crystalline unlabeled insulin. Thus, the phenomena seem to reflect the fate of all of the insulin within the system rather than merely the labeled material. However, some adsorptive peculiarities of the I^{131}-insulin due to modification of the phenolic hydroxyl in the iodinated tyrosyl groups cannot be wholly excluded.

C. Effects of human serum albumin on adsorption to glassware and spontaneous degradation. Similar protocols were employed to assess the effects of HSA upon the adsorption to glassware and spontaneous degradation of dilute insulin solutions. For these studies, open 20 ml. beakers were filled with 2.0 ml. of phosphate buffer (pH 7.4) containing *constant* amounts of labeled and unlabeled insulin (0.25 μg. insulin per ml.) and

TABLE II

Effect of human serum albumin upon adsorption of insulin to glassware and spontaneous degradation of insulin during incubation in aqueous media

Reaction mixture: 2.0 ml. pH 7.4 phosphate buffer containing 0.25 μg. insulin and 0 to 10.0 mg. HSA per ml. Incubation: 30 minutes, 38° C.

No.	Insulin	HSA	A*	B*	C*
	μg./ml.	mg./ml.			
1	0.25	0	46.8	21.1	5.3
2	0.25	0.05	16.3	6.4	2.0
3	0.25	0.10	8.7	2.3	0
4	0.25	0.20	7.9	2.1	0
5	0.25	0.50	6.1	1.6	0
6	0.25	1.00	5.9	1.6	0
7	0.25	2.00	6.2	1.4	0
8	0.25	5.00	4.3	0.8	0
9	0.25	10.00	4.4	0.7	0

* As in Table I.

varying amounts of HSA (0 to 10.0 mg. per ml.) (Table II). After 30 minutes of incubation of these beakers, the adsorption of radioactivity to glassware in vessels containing no HSA averaged 46.8 per cent as above (Table II, Column A). However, with as little as 0.10 mg. HSA per ml. in the suspending media, this adsorption was reduced to below 10 per cent. Moreover, when the initial suspending medium contained 0.50 mg. HSA per ml., two aqueous washings sufficed to recover more than 98 per cent of the radioactivity (Table II, Column B). Finally, the spontaneous degradation of insulin during 30 minutes of aerobic incubation did not occur in media containing 0.10 mg. HSA per ml. (Table II, Column C).

Additional studies were performed to evaluate the reversibility of glassware adsorption. More than half of the insulin adsorbed to glass during 30 minutes of incubation of aqueous media containing 0.25 μg. insulin per ml. could be recovered by introducing 25 mg. HSA into the system for 5 minutes prior to decanting.

Experiments to be published elsewhere would indicate that HSA does not contain insulin, and that the effects of HSA are not due to an interaction between HSA and insulin. Rather, at pH 7.4, insulin and HSA seem to compete for similar binding sites on the glass surface.

D. Conclusions. Significant adsorption to glassware and spontaneous degradation of insulin may occur during incubation of dilute insulin solutions in open vessels. The proportional magnitude of the phenomena at pH 7.4 is conditioned by *a*) the concentration of insulin, *b*) the presence of other negatively charged proteins, such as HSA, and *c*) the relationship between surface area and volume of medium. The contribution of such methodological artifacts can be minimized by the inclusion of HSA in all suspending media.[2]

2. Degradation of insulin by placental structures

Placental structures were prepared in various ways to assess their capacity to degrade insulin into TCA-soluble products.

A. Comparison of slices versus homogenates. Results of one of three experiments are presented in Table III. Slices of chorion, amnion, and placenta (200 ± 10 mg. wet weight), and 0.2 ml. aliquots of maternal or cord blood were incubated in 2 ml. KRP-Glucose containing unlabeled insulin and tracer quantities of I^{131}-insulin. Cell-free systems were prepared by homogenizing 1.0 Gm. of slices or 1 ml. of maternal or cord blood with 4 ml. of KRP-Glucose and then incubating 1 ml. aliquots of the homogenates with the same amount of insulin in a final volume of 2.2 ml. During 60 minutes of incubation, all of the slice systems liberated appreciable quantities of "nonprecipitable I^{131}" (Table III). Placental slices were most active on the basis of wet weight. However, none of the slice preparations was as active as homogenates prepared from equivalent amounts of tissue

TABLE III

The degradation of insulin by placental structures

Reaction mixture: KRP-Glucose, pH 7.4 plus 200 ± 10 mg. cellular preparation or 1 ml. of 1:5 homogenate. Final insulin concentration 25.02 μg. per ml. Incubation: 60 minutes, 38° C. For details, see text.

	Nonprecipitable I^{131} (% of total I^{131})	
	Slices	Homogenate
Tissue:		
Chorion	11.5	57.1
Amnion	10.6	51.3
Placenta	36.6	67.5
Maternal blood	1.3	0.1
Cord blood	0.5	0.1

[2] Many other proteins are equally effective. However, the concentrations of HSA which prevent adsorption to glassware and spontaneous degradation do not affect enzymatic proteolysis of insulin whereas some of the other proteins, such as Cohn Fractions III and IV-1, contain noncompetitive inhibitors of insulin-degrading enzymes (23).

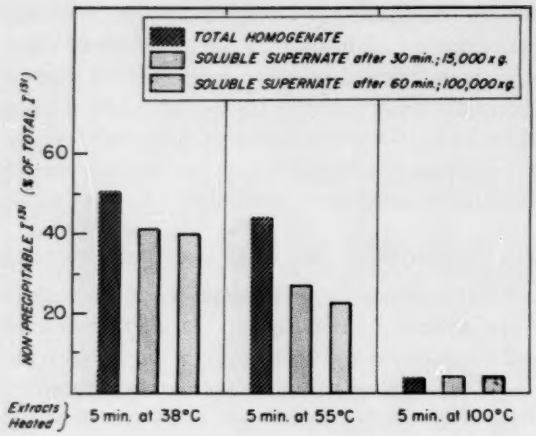

FIG. 1. INSULIN DEGRADATION BY SUBCELLULAR FRACTIONS OF HUMAN PLACENTA: THERMAL LABILITY OF SUBCELLULAR FRACTIONS

Placental fractions were prepared as outlined in text and heated for five minutes at 38°, 55° or 100° C. prior to incubation. Nitrogen contents of the 1:3 total homogenate, 15,000 × G and 100,000 × G soluble supernatants were 689.7, 455.6, and 336.4 mg. per 100 ml., respectively. Incubation: 30 minutes, 38° C. Insulin content of reaction mixture: 50.01 μg. per ml.

(Table III). The findings cannot be attributed to occluded blood since negligible degradation was effected by maternal or cord blood.

B. Subcellular localization of placental activity. A portion of a 1:3 placental homogenate was stored at 4° C. while other aliquots were centrifuged either in *a*) an International Refrigerated centrifuge at 4° C., 15,000 × G for 30 minutes to sediment cellular debris, nuclei and most mitochondria or in *b*) a Model L Spinco preparative ultracentrifuge at 4° C., 100,000 × G for 60 minutes to sediment most of the microsomes in addition to the denser subcellular components. Portions of intact homogenate and the two supernatant solutions were heated for 5 minutes in water baths at 38°, 55° and 100° C. Thereafter, 0.5 ml. aliquots of the various heated and unheated preparations were incubated for 30 minutes at 38° C. with 1.5 ml. of insulin-containing phosphate buffer. As shown in Figure 1, most of the insulin-degrading capacity of human placenta was localized in the soluble cytoplasm which remained after centrifugation at 100,000 × G. Specific activity expressed as "per cent nonprecipitable I^{131} per mg. N of placental extract" was increased 60 to 200 per cent by such centrifugation. The activity was largely, although not entirely, destroyed by 5 minutes of boiling.

C. Conclusions. Human placenta contains heat-stable and heat-labile systems for liberating TCA-soluble radioactivity from I^{131}-insulin. Activity is maximal in cell-free systems and localized principally in the nonparticulate soluble cytoplasm.

3. Products of insulin degradation by placental extracts

Additional techniques were employed to characterize the products of the reaction between insulin and placental extracts. For these and for all subsequent studies, placental homogenates were prepared and incubation was performed according to the *standard experimental procedure.*

A. Paper electrophoresis. In dilute solution, I^{131}-insulin remains bound at the origin during paper electrophoresis in veronal buffer at pH 8.6 (14, 21, 24). However, paper electrophoresis of the reaction mixture following incubation of I^{131}-insulin and placental extract disclosed radioactive products which migrated in the areas corresponding to the slowest moving portion of albumin and the α_2-globulins (Figure 2). In addition, occasionally a small amount of radioactivity was noted in the prealbumin area. More than 90 per cent of the applied radioactivity could be accounted for in these areas. Under similar conditions, recovery of inorganic I^{131} averages 3.1 per cent (16). When multiple reaction vessels were prepared with constant tracer quantities of I^{131}-insulin and varying amounts of crystalline unlabeled insulin, the formation of radioactive degradation products was inversely proportional to the total amount of insulin within the system (Figure 2). Although the characteristic electrophoretic migration at pH 8.6 of the radioactive degradation products may have resulted in part from associations with plasma proteins, a similar migration was exhibited during electrophoresis of the supernatant solutions obtained following TCA-precipitation of the reaction mixtures.

B. Paper chromatography. In both acid (BAc) and alkaline (BDA) solvents, the radioactivity in the reaction mixture at all intervals following incubation was apportioned between the origin and areas which corresponded to the migration of the

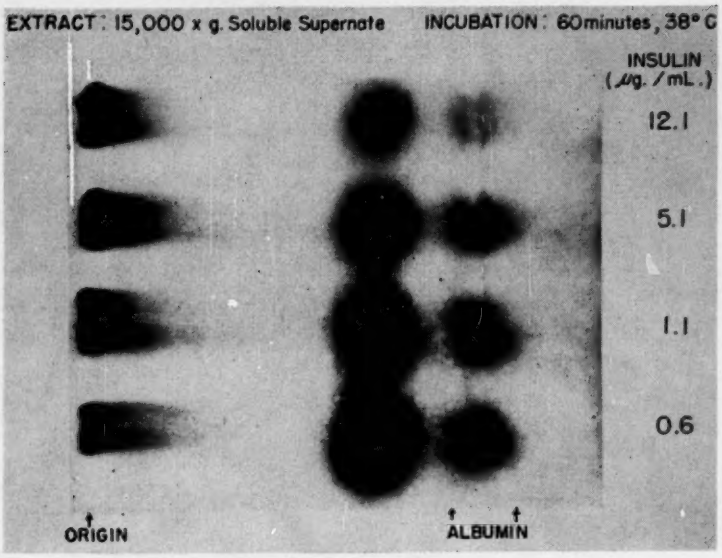

FIG. 2. ELECTROPHORETIC MIGRATION OF PRODUCTS OF I^{131}-INSULIN DEGRADATION BY PLACENTAL EXTRACT

Standard 15,000 × G placental extract was diluted fivefold with pH 7.4 phosphate buffer to yield the equivalent of 16.6 mg. wet weight placenta per ml. final reaction mixture. Final insulin concentration was as indicated in illustration. Incubation: 60 minutes, 38° C. Reaction mixture directly applied for paper electrophoresis at end of incubation. Electrophoresis was performed at 4° C. to minimize further activity of the dilute enzyme preparation. Radioautograph of electrophoretograms is depicted above.

MIT and DIT markers (Figure 3). The ratios of $\frac{\text{"MIT-like"}}{\text{"DIT-like"}}$ radioactivity ranged from 3.0 to 4.5 with four preparations of I^{131}-insulin. Negligible radioactivity was observed in the zones corresponding to ionic iodide. Further characterization of the radioactive products was not attempted since it is not germane to the present studies. Thus, whether the "MIT- and DIT-like" components consist entirely of iodinated tyrosines (and do not include peptides of similar mobilities), and whether all of the "origin" radioactivity represents unaltered I^{131}-insulin cannot be said with certainty. However, from the data, simple deiodination may be excluded as the basis for the manifest placental activity. Since the absolute formation of radioactive "MIT- and DIT-like" products was reduced by the inclusion of increasing quantities of unlabeled insulin in the reaction mixtures (Figure 3), it was inferred that the I^{131}-insulin validly mirrored the proteolysis of unlabeled insulin.

C. Liberation of TCA-soluble products of optical density at 280 mμ. Documentation of proteolysis of unlabeled insulin was secured by the method of Tomizawa and Williams (25). Homogenates were prepared by the *standard experimental procedure* and dialyzed for 18 hours at 4° C. against repeated changes of 0.1 M phosphate buffer, pH 7.4. Thereafter, 1 ml. aliquots were incubated with 1 ml. of *a*) phosphate buffer or *b*) phosphate buffer containing 2 mg. crystalline insulin per ml. Control vessels containing phosphate buffer alone or phosphate buffer plus insulin were similarly incubated. At various times during incubation, the contents of the beakers were quantitatively decanted into 3 ml. of chilled 10 per cent TCA, and the TCA-soluble supernatant solutions were read in a Beckman DU spectrophotometer at 280 mμ. Significant evolution of TCA-soluble, ultraviolet-absorbing moieties was only observed in systems containing both insulin and placental extract (Table IV).

The preliminary dialysis in these studies was instituted to reduce the appreciable concentration

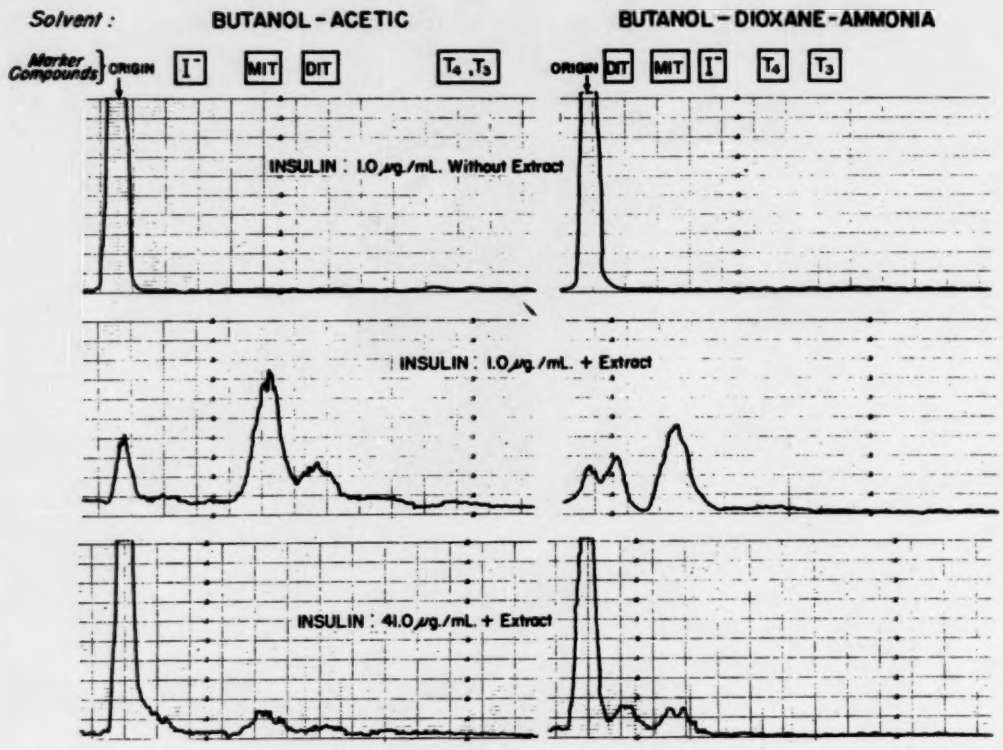

FIG. 3. CHROMATOGRAPHY OF PRODUCTS OF I^{131}-INSULIN DEGRADATION BY PLACENTAL EXTRACT

Reaction mixture: 2 ml. pH 7.4 phosphate buffer containing 15,000 × G placental extract as in *standard experimental procedure.* Incubation: 30 minutes, 38° C. At end of incubation, aliquot of reaction mixture was applied to Whatman No. 1 filter paper, supplemented with carrier compounds, dried and chromatographed as indicated in text. Radioactive scans of chromatograms are illustrated above.

of nonprecipitable components of optical density at 280 mμ in the placental extracts. However, such dialysis also diminishes the insulin-degrading capacity of placental preparations.

TABLE IV

Liberation of TCA-soluble products of optical density at 280 mμ.

Reaction mixture: 2 ml. pH 7.4 phosphate buffer ± crystalline, unlabeled insulin in final concentration of 1 mg. per ml. ± dialyzed 15,000 × G placental extract as in *standard experimental procedure.* Incubation: 38° C.

Phosphate buffer plus	Optical density at 280 mμ*		
	0 min. incubation	30 min. incubation	60 min. incubation
Phosphate buffer	0.075	0.070	0.072
Insulin	0.080	0.075	0.078
Placental extract	0.330	0.334	0.350
Insulin plus placental extract	0.328	0.410	0.535

* Reaction mixture was decanted into TCA; optical density of TCA supernatant was determined.

D. Bioassay. Alteration in the biological potency of insulin incubated with placental extracts was assessed by injecting portions of control and reaction mixtures into the ear veins of fasted 2 to 3 Kg. rabbits. Timed blood specimens for glucose analysis were obtained from the vessels of the ear or by cardiac puncture. Each solution was assayed in a separate animal. One of four experiments is presented in Table V. Insulin which had been incubated for 30 minutes with placental extract did not produce the same degree of hypoglycemia as insulin which had been incubated for a comparable period of time but without placental extract. The placental extracts *per se* effected negligible changes in blood sugar levels.

E. Conclusions. The electrophoretic, chromatographic and spectrophotometric studies demon-

TABLE V

Inactivation of insulin by placental extracts

Reaction mixture: 2.0 ml. pH 7.4 phosphate buffer containing 15,000 × G placental extract as in *standard experimental procedure* and 60 μg. (1.5 units) insulin per ml. Control vessels containing a) placental extract without insulin, and *b*) insulin without placental extract, were incubated concurrently. Incubation: 30 minutes, 38° C.

Assay: Rabbits were injected with 1.0 ml. of reaction mixture at end of incubation.

Reaction mixture	Minutes after injection (% of initial blood sugar)	
	30 min.	*60 min.*
Phosphate buffer plus insulin	42.4	56.8
Phosphate buffer plus placental extract	95.1	98.9
Placental extract plus insulin	62.1	80.1

strate that the formation of TCA-soluble radioactivity from I^{131}-insulin by placental extracts represents proteolysis of the insulin molecule. The biological consequence of this proteolysis is evidenced by a reduction in the hypoglycemic potency of the insulin.

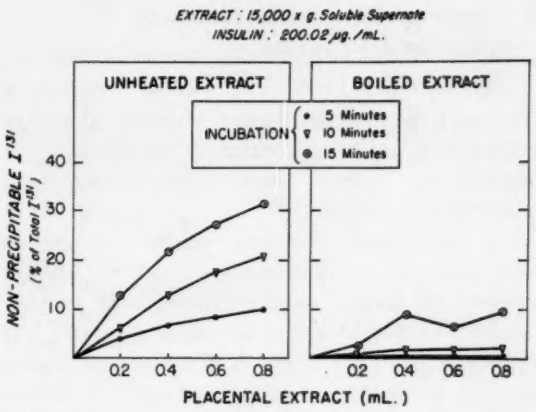

FIG. 4. RELATIONSHIP BETWEEN QUANTITY OF PLACENTAL EXTRACT AND INSULIN DEGRADATION

15,000 × G placental extract was prepared as in *standard experimental procedure*. Various quantities of unheated and boiled extract were incubated for 5, 10 or 15 minutes in a reaction mixture consisting of 2.0 ml. volume and containing 200.02 μg. insulin per ml.

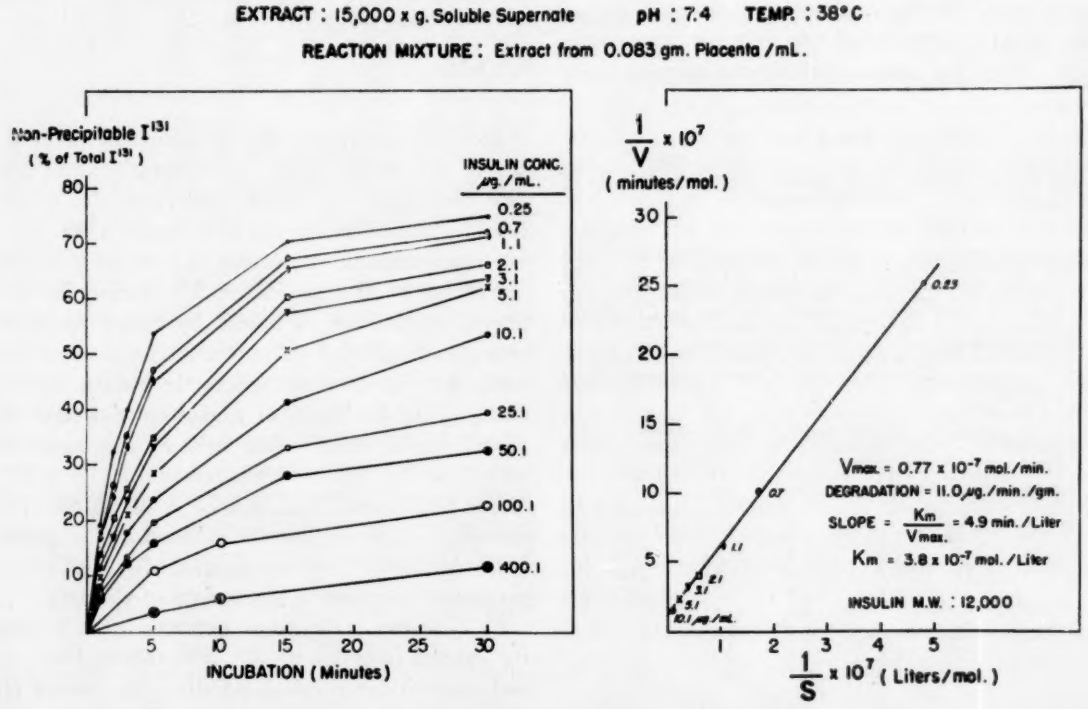

FIG. 5. KINETICS OF INSULIN DEGRADATION BY PLACENTAL EXTRACTS

Reaction mixture: 2 ml. pH 7.4 phosphate buffer containing various quantities of insulin and 15,000 × G placental extract was prepared as in *standard experimental procedure*. Aliquots of reaction mixtures were removed at various intervals during incubation. Nitrogen content of the 1 : 3 15,000 × G placental extract employed for this study was 408.2 mg. per 100 ml.

4. Characterization of the insulin-degrading systems of human placenta

Although the 15,000 × G soluble supernatant fraction derived from human placentas does not represent a purified preparation, its activity was assessed by various conventional techniques of enzymology.

A. Relationship between insulin degradation and quantity of placental extract. Constant amounts of insulin were incubated with varying amounts of the 15,000 × G placental extract. A representative experiment is illustrated in Figure 4. The formation of nonprecipitable I^{131} during incubation of I^{131}-insulin was directly correlated with the quantity of extract in the reaction mixture. Such a relationship did not obtain with extract which had been boiled for 5 minutes prior to incubation.

B. Kinetics. The left-hand portion of Figure 5 represents the fraction of I^{131}-insulin degraded as a function of time in the *standard experimental procedure.* Prolonged linear degradation was not observed at any substrate concentration. Thus, for kinetic analysis, the initial velocity at each concentration was determined as the product of the initial insulin concentration and the zero time slope. A Lineweaver-Burk plot of the values obtained during the first minute at the lowest concentrations of insulin is presented in the right-hand portion of Figure 5. During this interval, degradation of insulin by boiled placental extracts is negligible and correction for the thermostable activity does not affect the values significantly. On the basis of a molecular weight of 12,000 for insulin, the Michaelis-Menten constant (Km) for the thermo-labile reaction is 3.8×10^{-7} Moles per L. and V_{max} is 0.77×10^{-7} Moles per minute. Correction for the dilution in the *standard experimental procedure* yields a V_{max} of 11 μg. per minute per Gm. of wet weight of placenta.

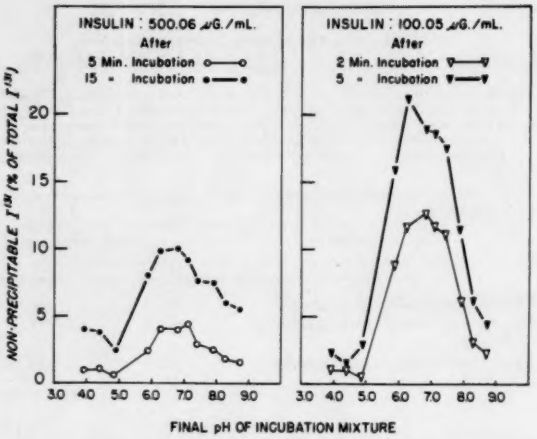

FIG. 6. THE EFFECT OF pH ON INSULIN DEGRADATION BY PLACENTAL EXTRACT

Reaction mixture containing 2 ml. volume and 15,000 × G placental extract as in *standard experimental procedure.* Acetate buffer employed for pH 3.5 to 4.5; phosphate buffer for pH 5.4 to 8.1; and Tris (hydroxymethyl) aminomethane buffer for pH 8.2 to 9.0 The pH values of the reaction mixtures before and after 15 minutes of incubation were: 3.50, 3.97; 4.00, 4.40; 4.57, 4.87; 5.41, 5.91; 6.00, 6.23; 6.65, 6.84; 7.00, 7.15; 7.40, 7.42; 8.10, 7.91; 8.20, 8.27; and 9.00, 8.71.

TABLE VI

Thermal inactivation of the insulin-degrading capacity of placental extracts

Reaction mixture: aliquots of 15,000 × G placental extract were heated at various temperatures (38°, 50°, 60° and 80° C.) for varying intervals (5, 10 and 20 minutes) prior to incubation. Thereafter, incubation was performed at 38° C. with 2 ml. pH 7.4 phosphate buffer containing 100.1 μg. insulin per ml. and *treated* placental extract as in *standard experimental procedure.*

Insulin degradation was assessed by sampling from reaction vessels after 1, 5, 15 and 30 minutes of incubation and corrected for TCA-soluble radioactivity in control vessels incubated without extract.

Preincubation heating of extract		Nonprecipitable I^{131} (%) formed during incubation of extract for:			
Temp.	Duration	1 min.	5 min.	15 min.	30 min.
° C.	*min.*				
38	5	7.2	14.6	15.1	22.9
	10	7.4	12.9	15.8	21.8
	20	6.8	11.2	15.0	22.2
50	5	6.1	11.3	14.5	21.6
	10	5.6	11.1	13.2	22.1
	20	4.4	8.7	10.8	18.2
60	5	7.8	7.8	9.1	14.4
	10	3.3	6.1	7.5	12.9
	20	3.2	3.9	7.2	8.9
80	5	0.7	2.9	3.5	6.7
	10			3.4	7.0
	20	0.5	1.6	3.6	6.2

C. Stability. Placental extracts were heated for various intervals at 38°, 50°, 60° and 80° C. and assayed for residual activity. As shown on Table VI, about one-fourth of the insulin-degrading capacity which was observed during 15 to 30 minutes of incubation remained, even after 20 minutes of heating at 80° C.

The thermo-labile enzymatic activity was re-

duced by storing 15,000 × G placental extracts at − 18° C. for prolonged periods. Kinetic analysis indicated that this resulted from a diminution of V_{max} without a significant change in Km. The thermo-stable activity was not reduced by storage of frozen placental extracts.

D. Effect of pH and temperature. The relationship between pH and enzymatic activity is shown in Figure 6. Experiments were conducted with short incubation to minimize the contributions of the relatively slow, thermo-stable component. At all insulin concentrations, insulin degradation was maximal between pH 6.5 and 7.0.

The relationship between insulin degradation and temperature of incubation is demonstrated in the experiment presented in Table VII. During 5 minutes of incubation, the mean temperature velocity constant was 22,000 cal. per Mole for the range 24° to 34° C. and 9,000 cal. per Mole for 34° to 38° C. Reaction velocity declined at temperatures between 42° and 55° C.

E. Inhibition of enzymatic activity. Enzyme inhibitors previously assessed in the "insulinase" system of rat liver (10, 12, 26) were examined with placental extracts (Table VIII). Profound inhibition was effected with reagents which may affect sulfhydryl groups by oxidation-reduction (cf., cupric salts), alkylation (cf., iodoacetate), or mercaptide formation (cf., *p*-chloromercuribenzoate).

Specificity of the placental system was assessed by incubating small quantities of insulin (5×10^{-7} M)[3] in the presence of 1×10^{-6} to 1×10^{-5} M[3] concentrations of crystalline bovine serum albumin,[1] or protein hormones such as glucagon,[1] corticotropin (ACTH),[1] thyrotropin (TSH),[1] somatotropin (STH),[1] and lactogenic hormone (LTH).[1] Reactions were terminated after 5 minutes of incubation to minimize the inhibitory potential of products of proteolysis. At concentrations of 1×10^{-6} M, none of the proteins appreciably altered the degradation of insulin (Figure 7). When present in 20-fold excess, the protein hormones effected a modest reduction in the rate of insulin breakdown. This was most pronounced with ACTH (Figure 7). Unlabeled insulin, added to a final insulin concentration of 1×10^{-6} to 1×10^{-5} M, more effectively reduced the formation of nonprecipitable I^{131} than any other protein (Figure 7).

Despite the relatively small effects of the protein hormones, the potential physiological significance of the interrelationships prompted further examination of the inhibition. Results of such studies with glucagon and ACTH are shown in Figure 8. During 30 minutes of incubation at multiple concentrations, the effects of ACTH and glucagon conformed to a competitive type of inhibition.

F. Conclusions. The rapid degradation of insulin by unboiled placental extracts exhibits the lability, kinetic characteristics, thermal relation-

TABLE VII

Insulin degradation by placental extracts at various temperatures of incubation

Reaction mixture: 2.0 ml. pH 7.4 phosphate buffer containing unheated or boiled 15,000 × G placental extracts as in *standard experimental procedure* and 25.01 μg. insulin per ml. Reaction was terminated after 5 minutes of incubation. Values for nonprecipitable I^{131} were corrected for control vessels incubated without placental extract at identical temperatures.

Incubation temp.	Nonprecipitable I^{131} (% of total I^{131})	
	Unheated extract	Boiled extract
° C.		
24.5	4.1	0
30.0	8.4	0.1
33.8	12.9	0.3
38.0	16.0	0.3
42.0	16.9	0.1
55.2	9.9	1.2
79.0	0.1	0.1

TABLE VIII

The effect of enzyme inhibitors on insulin degradation by placental extracts

Reaction mixture: 2 ml. pH 7.4 phosphate buffer containing 100.03 μg. insulin per ml., 15,000 × G placental extract as in *standard experimental procedure* ± enzyme inhibitor. Incubation: 30 minutes, 38° C.

Inhibitor	Concentration (× 10^{-3} M)	Nonprecipitable I^{131} (% of total I^{131})
None		31.8
p-Chloromercuribenzoate	0.5	2.9
Cupric sulfate	0.5	5.4
Zinc sulfate	0.5	25.1
Iodoacetic acid	1.0	18.8
Sodium azide	1.0	30.3
Sodium arsenite	1.0	27.3

[3] Calculations of molar concentrations were based on the following values for molecular weight: insulin, 12,000; bovine serum albumin, 70,000; glucagon, 4,200; corticotropin, 4,000; thyrotropin, 10,000; somatotropin, 44,250; and lactogenic hormone, 32,000.

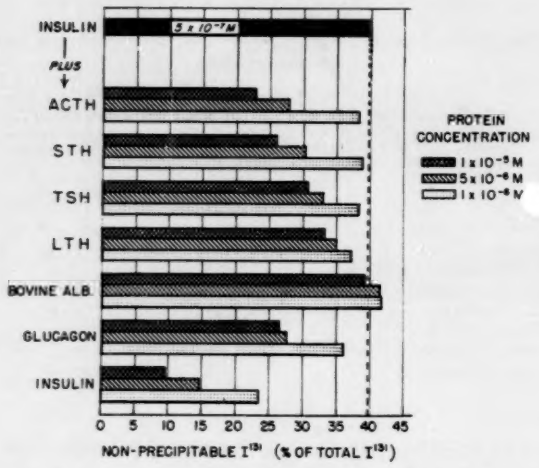

FIG. 7. THE EFFECT OF VARIOUS PROTEINS ON INSULIN DEGRADATION BY PLACENTAL EXTRACTS

Incubation: 5 minutes, 38° C. For details see text.

ships and circumscribed pH dependencies that are typical of enzymatic reactions. Moreover, the activity is impaired by sulfhydryl reagents and may be competitively inhibited in the presence of large quantities of such protein hormones as glucagon or ACTH. Contrariwise, the degradation of insulin by boiled placental extracts does not appear to be enzymatically mediated. The activity is slow, stable, and relatively uninfluenced by variation in the quantity of placental extract or by the temperature of incubation.

5. Normal values for insulin degradation by human placentas

In order to establish values for the normal insulin-degrading capacity of human placenta and to obtain control data for future studies of abnormal pregnancies, nine term placentas were assayed on separate days with multiple concentrations of insulin and the *standard experimental procedure*.

Analyses were obtained following 5 and 30 minutes of incubation. Five different preparations of I^{131}-insulin were employed in the nine studies. Values for fractional insulin degradation in systems containing 25, 50, 100 and 400 μg. of crystalline insulin per ml. are presented in Figure 9. Significant differences could not be demonstrated in the insulin-degrading capacities of placentas obtained by vaginal delivery and placentas obtained at elective Caesarian section prior to the inception of labor. Nitrogen content of the 15,000 × G supernatant solutions derived from these 1:3 placental homogenates averaged 423.9 mg. N per 100 ml. ± 73.1.[4] For a single experiment, a placenta was obtained at the spontaneous abortion of an 18 week old fetus. An extract prepared from this placenta by the *standard experimental procedure* contained 233.2 mg. N per 100 ml. and effected distinct degradation of insulin (Figure 9).

For purposes of comparison, four similar experiments were performed with livers from fed male rats. Mean values for insulin degradation by the 15,000 × G supernatant solutions of 1:3 rat liver homogenates are shown in Figure 9. The insulin-degrading capacities of the rat liver preparations exceeded those of comparably prepared placental extracts. However, since the nitrogen content of the four rat liver extracts averaged 828.9 mg. per 100 ml., the differences in activities are not as great when expressed on a nitrogen rather than on a wet weight basis.

Conclusions. The studies with the nine term placentas indicate that ranges of normal activity

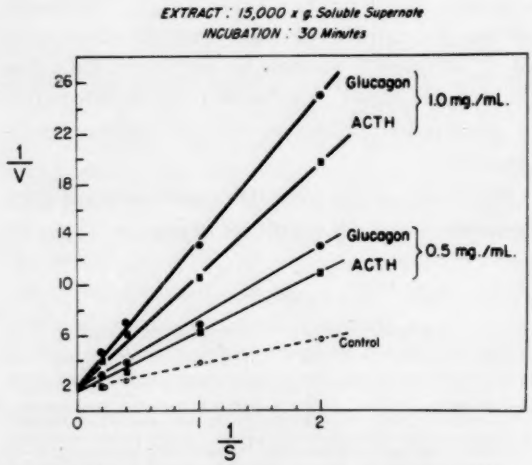

FIG. 8. THE NATURE OF THE INHIBITION OF PLACENTAL INSULIN DEGRADATION IN THE PRESENCE OF ACTH OR GLUCAGON

Reaction mixture: 2.0 ml. pH 7.4 phosphate buffer containing 20.2, 40.2 or 100.2 μg. insulin per ml., 15,000 × G placental extract as in *standard experimental procedure*, and ± 0.5 or 1.0 mg. ACTH or glucagon per ml. V = units of I^{131}-insulin degraded in 30 minutes of incubation at 38° C. as judged by the evolution of nonprecipitable I^{131}. S = units of insulin per ml. of reaction mixture.

[4] Mean ± standard deviation.

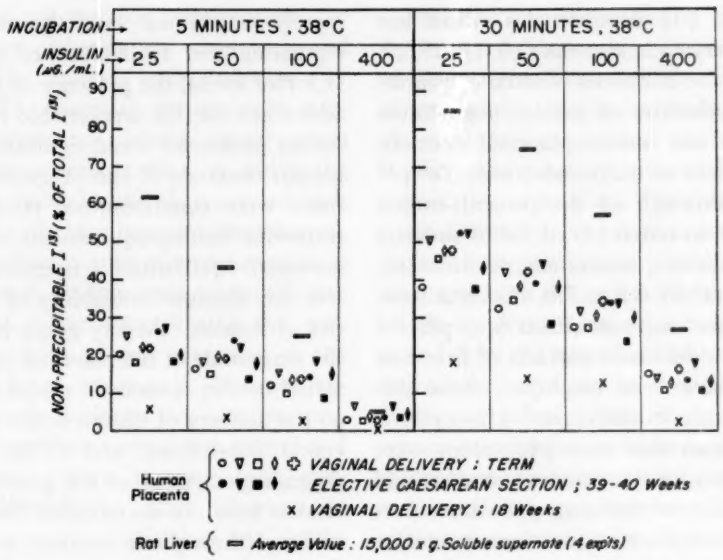

FIG. 9. THE DEGRADATION OF INSULIN BY EXTRACTS OF HUMAN PLACENTA AND LIVERS OF FED MALE RATS

Details of incubation and preparation of extracts are as in *standard experimental procedure*. Values for placentas were derived from individual experiments. Cross-hatched bars refer to mean values of four experiments with 15,000 × G rat liver extracts.

are narrow and that the measurement of insulin-degrading capacity represents a reproducible parameter of human placental function. The similar potencies of both Caesarian section and "vaginal delivery" placentas would suggest that insulin degradation does not result from an activation of proteolytic mechanisms during labor. Rather, the manifest activity of the single 18 week placenta may constitute evidence that placental mechanisms for insulin degradation are present at least as early as the second trimester.

DISCUSSION

The present studies have provided the first detailed *in vitro* characterization of an insulin-degrading system derived from human tissue. It has been demonstrated that cellular and subcellular preparations of human placenta can degrade I^{131}-insulin into radioactive products which are soluble in TCA, electrophoretically distinct from ionic iodide, and which approximate the chromatographic mobilities of iodinated tyrosines. This evidence for the proteolysis of I^{131}-insulin has been corroborated and extended with unlabeled crystalline insulin. Thus, it has been shown that TCA-soluble, ultraviolet-absorbing products of proteolysis are evolved during incubation of native insulin with human placental extracts. Concomitantly, there is an attenuation of the hypoglycemic potency of the insulin.

Inactivation of insulin by extracts of certain tissues of animal origin has been known for some time (26–29). With the advent of I^{131}-insulin, the mechanisms have been investigated and characterized perhaps most extensively in rat and beef liver preparations (9–12, 20, 25, 30, 31). The similarities between the published findings for animal liver "insulinase" and the insulin-degrading system in human placenta are striking. Liver "insulinase," like the placental activity, *a*) is localized in the soluble cytoplasm during subcellular fractionation (20, 29, 31, 32); *b*) is inhibited by sulfhydryl reagents (10, 12, 26); *c*) is reduced by dialysis or storage (28, 29, 33); and *d*) contains both heat-stable as well as heat-labile components (10, 20, 26, 28, 32). Moreover, in animal liver as in human placenta, the heat-labile component displays the activation energy, reaction kinetics

and circumscribed pH dependencies which are characteristic of enzymatic systems (10, 12, 25, 29, 30, 34). Indeed, the Michaelis-Menten constants, based on initial velocities of insulin degradation by rat liver (34) and human placental extracts, are of similar orders of magnitude; only the pH optima differ. Although all the present studies were conducted at an initial pH of 7.4 to simulate physiological conditions, maximum placental activity is obtained at pH 6.5 to 7.0. Contrariwise, rat liver "insulinase" is most effective at pH 7.6 to 8.0 (10). With the crude extracts of liver and placenta which have been employed, these differences are difficult to interpret. However, it should be mentioned that final pH values were not recorded in any of the previous studies, and hence, the possibility of declining pH values during incubation of rat liver preparations cannot be excluded.

The available data do not justify conclusions regarding the substrate specificity of the heat-labile placental system, nor can it be stated whether one or several enzymes participate in the manifest cleavage of insulin. Relative specificity is indicated by the fact that equimolar concentrations of a variety of proteins did not appreciably modify reaction kinetics.[5] For the moment, definitive dissection of the insulin-degrading system of human placenta must await the results of further purification.

Regardless of its biochemical specificity, the physiological implications of this system warrant consideration. The placenta must be regarded, at least, as yet another tissue which can both respond to insulin and degrade insulin. For the placenta, as for other such tissues, it remains to be demonstrated whether local hormonal degradation is linked to insulin action or whether degradation merely conditions local hormonal activity by regulating the availability of unaltered insulin. In either event, the presence of the placenta as an added site for the irreversible removal of insulin during pregnancy could significantly alter the total insulin demands of the pregnant female. Unless there were commensurate reduction in the extrauterine insulin requirements, the maintenance of hormonal equilibrium in pregnancy would necessitate the absolute availability of increased quantities of insulin. At any given time in pregnancy, the magnitude of the placental contribution to maternal insulin economy[6] would be conditioned by *a*) the delivery of insulin to the placenta (*i.e.*, placental blood flow), and *b*) the potential insulin-degrading capacity of the placenta. The present studies have not documented the level of placental activity throughout human gestation, although manifest degradation was observed with the single 18 week placenta that was available for examination. Concurrent experiments with rats have demonstrated that proteolysis of insulin can be effected by the earliest formed rat placenta and that the activity per milligram of nitrogen in placental extracts remains constant throughout pregnancy (37). Thus, if rat data may be extrapolated to humans, it may be inferred that the contributions of placental insulin degradation to maternal insulin requirements should parallel *a*) the vascularity of the placenta, and *b*) the total functioning mass of placental tissue. With this in mind, review of the clinical features of the diabetogenic effects of pregnancy may be appropriate.

In human pregnancy, tolerance to intravenous glucose is not significantly altered (38) although the response to exogenous insulin is diminished, especially in the latter half of gestation (33). Presumably, therefore, an increased endogenous secretion of insulin is required for the preservation of normal carbohydrate metabolism during pregnancy. This premise is supported by the clinical sequence in subjects with diminished pancreatic reserve. Thus, frank diabetes mellitus frequently supervenes in prediabetic females during the latter phases of pregnancy, and, despite some conflicting

[5] The competitive inhibition observed with large quantities of ACTH and glucagon need not indicate a true substrate competition for the insulin-degrading system. In 18,000 × G extracts of rat liver, ACTH and glucagon also retard insulin breakdown (25). However, the hepatic enzymes for degrading ACTH and glucagon may be different from "insulinase" (35, 36) and "insulinase" in 18,000 × G rat liver extracts may be competitively inhibited by products of the proteolysis of ACTH or glucagon (30). Thus, if separate enzymes for ACTH and glucagon proteolysis are also present in the 15,000 × G placental extracts, then similar interrelationships might occur.

[6] Since there are at present no experimental data relevant to the fetal elaboration or the transplacental passage of insulin in primate pregnancy, these factors have been omitted from the above theoretical considerations.

reports (39), the maintenance requirements for insulin of pregnant diabetics are often increased at this time (4–8). Indeed, both Pedersen (5) and Bergqvist (7) observed the greatest augmentation of maintenance insulin requirements during the seventh month, a time at which the placenta is nearly fully developed, senescent vascular changes in the placenta have not yet occurred, the heightened cardiac output of pregnancy is at peak level (40), and, by inference, placental perfusion is maximal. The expulsion of the conceptus is followed by a prompt reversion of the diabetogenic effects of pregnancy. Insulin requirement of diabetic patients may decline precipitously in the immediate postpartum period and normoglycemia may return in prediabetic subjects (4–8). Although the latter phenomenon can be attributed in part to diminished food intake and the heightened muscular effort of labor, studies with intact rats in this laboratory would indicate that a true alteration in insulin metabolism occurs (37). Near term, in the rat, there is an acceleration in the fractional rate of turnover of labeled insulin which cannot be explained on the basis of transplacental loss of intact insulin, and which disappears within hours following parturition. Thus, from all the clinical evidence, some correlation does seem to obtain between insulin requirements and the presence and the level of function of an intrauterine insulin-degrading site.

However, alternative mechanisms could also explain these clinical findings. Among the extrauterine possibilities that have been suggested, the case for contra-insulin factors of pituitary origin is somewhat weakened by the recent studies of a woman who was hypophysectomized during pregnancy (41). In this patient, while on constant thyroid and cortisone replacement therapy, glucose tolerance was abnormal and response to intravenous insulin was diminished at 31 weeks of gestation whereas both tests were normal 5 days postpartum. Similarly, the case for adrenal contra-insulin factors is hard to reconcile with the fact that levels of blood glucocorticoids remain elevated in the immediate postpartum period (42) despite the occasional acute reduction in insulin requirements of diabetics. As a matter of fact, the whole concept of increased adrenocortical activity during pregnancy has recently been challenged (43).

From all considerations, it would appear that the temporal relationships are most consonant with an intrauterine basis for the diabetogenic effects of pregnancy. Mediation could be effected by *a*) placental (or even fetal) elaboration of contra-insulin factors or *b*) placental (or even fetal) degradation of insulin. The present studies do not aid in differentiating between the secretory and the degradatory possibilities. Insofar as mechanisms have now been demonstrated for the proteolysis of insulin by placenta, an experimental basis has been provided for the latter possibility.[7] However, such degradation need not be confined to insulin. By analogy to other tissues, the placenta might also contain enzymatic mechanisms for the proteolysis of glucagon or pituitary principles which affect pancreatic cell activity. Moreover, the alternatives of intrauterine secretion or degradation need not be mutually exclusive. It is doubtful whether any metabolic change in as complex an event as mammalian pregnancy can ever be explained in terms of a single mechanism. From the present data, it may only be suggested that alterations in hormone economy secondary to hormone degradation within the conceptus warrant consideration in any analysis of gestational changes in carbohydrate metabolism.

SUMMARY

1. The presence of a system for the proteolytic inactivation of insulin has been demonstrated in human placental tissue.

2. During incubation of insulin with cellular and subcellular preparations of human placenta, insulin is degraded into trichloroacetic acid-soluble, ultraviolet-absorbing products of proteolysis. Concomitantly, the hypoglycemic potency of insulin is attenuated.

3. The system has been characterized with I^{131}-insulin and cell-free placental extracts. It has been demonstrated that placental insulin-degrading activity is localized within the soluble, nonparticu-

[7] The placental insulin-degrading system may also provide a means of studying one of the suggested etiologies of diabetes mellitus. Mirsky has postulated that increased degradation of insulin could antedate manifest diabetes mellitus and constitute the insulinogenic challenge in the prediabetic state (29, 30). The placenta may be proposed as an easily accessible human tissue wherein this thesis may be experimentally evaluated in subjects with large babies or other prediabetic stigmata.

late cytoplasm and consists of heat-stable and heat-labile components. The heat-labile activity effects most of the rapid inactivation of insulin and exhibits properties that are characteristic of enzymatic mechanisms. At 38° C., and pH 7.4, the Michaelis-Menten constant (Km) for the reaction was found to be 3.8×10^{-7} Moles per L., and V_{max} was 11.0 μg. per minute per Gm. of wet weight of placenta.

4. The activities of multiple normal term placentas were assayed at various concentrations of insulin in order to obtain control data for future studies of abnormal pregnancies. It was demonstrated that insulin-degrading capacity constitutes a reproducible parameter of human placental function. Manifest activity was displayed by a single placenta obtained at the spontaneous abortion of an 18 week old fetus.

5. The insulin-degrading system of human placenta has been compared to liver "insulinase" preparations of animal origin.

6. It has been suggested that proteolytic inactivation of protein hormones within the conceptus may be of physiological significance in the gestational changes in carbohydrate economy.

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BILE PIGMENTS OF JAUNDICE *

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Current concepts concerning the formation and metabolism of bile pigments hold that bilirubin is formed from the catabolism of hemoglobin by the reticuloendothelial system, mainly in bone marrow and spleen, and that the bilirubin is then transported to the liver where it is modified and excreted via the biliary system. These concepts are based largely on the studies by Mann, Magath and Bollman (1, 2) of the effect of total hepatectomy on mammals which show that after complete extirpation of the liver, indirect-reacting bile pigment was formed and accumulated in the serum progressively during the survival period of the animal. Bollman and Mann (3) noted that as the amount of bilirubin in the blood increased after hepatectomy, the van den Bergh reaction changed from an indirect to a direct reaction and bilirubin began to appear in the urine.

Since the original work of van den Bergh, Snapper and Muller (4, 5) on the application of the Ehrlich diazo reaction to the quantitative measurement of the serum bilirubin and their observation of the differing character of the reaction in various types of jaundice, many investigators have sought to determine the fundamental basis for these differences. Despite a voluminous literature on the subject which has accumulated for more than 40 years, disagreement and lack of exact knowledge are still evident. In this paper we can cite only recent surveys encompassing the many concepts which have been proposed (6–9).

Recently the studies of Cole and Lathe (10) have provided a new and important approach to these problems. By the technic of reverse phase chromatography using silicone-treated kieselguhr, these workers were able to separate from protein-free extracts of icteric serum a nonpolar, indirect-reacting pigment which in all its characteristics resembled pure crystalline bilirubin. Also isolated was a polar, direct-reacting pigment which differed from the indirect-reacting bilirubin in being water soluble and in spectroscopic absorption.

With the use of a different solvent system, these same workers with Billing demonstrated that the direct-reacting fraction could be separated into two pigments which they termed "I and II" (11). Evidence was presented, based on the differing chromatographic behavior of the diazotized pigments, that Pigment I appeared to represent an intermediate structure between the indirect-reacting pigment and Pigment II (12).

More recently, Billing and Lathe (13) showed that Pigment II is the diglucuronide of bilirubin and that Pigment I is probably the monoglucuronide form. Their work has been confirmed and amplified (14–16). Recently, sulfate conjugates of bilirubin have been demonstrated in animal bile. It has been proposed that a small percentage of bilirubin is normally excreted in this form (17). The chromatographic methods used in this study (see below) do not distinguish the sulfate fraction. The entire subject has been thoroughly reviewed recently (18).

In light of this new and fruitful approach to the chemistry of bilirubin, we decided that some of the concepts of bilirubin metabolism as well as certain clinical aspects of the problem of jaundice should be re-examined by the technic of reverse phase partition chromatography. The work presented in this paper includes studies of *1*) the pigment composition of bile of the normal human, dog and rat; *2*) serum and urinary pigments in the dehepatized dog and rat; *3*) fistula bile in the dog and rat following injections of hemoglobin and bilirubin; *4*) serum pigments in animals with ex-

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perimentally produced extrahepatic biliary obstruction; *5*) serum pigments in animals with hepatocellular necrosis induced by carbon tetrachloride and by ethionine; *6*) serum pigments in 147 patients with hepatocellular and extrahepatic obstructive jaundice, including chromatography of the bile in 12 patients with hepatocellular jaundice; *7*) serum pigments in patients with various other types of jaundice; *8*) serum and biliary pigments in eight patients with constitutional hepatic dysfunction (Gilbert's disease); and *9*) the serum and bile pigments in the homozygous, congenitally jaundiced rat (Gunn strain).

METHODS USED IN STUDY OF BILE PIGMENTS

Kieselguhr was prepared according to the method of Howard and Martin (19). For the solvent systems and preparation of samples of blood, bile and urine, the methods of Cole, Lathe and Billing (11) were employed, with one exception. This was concerned with the preparation of serum from dehepatized dogs in which it was characteristically difficult to redissolve the dried protein-free serum pigment extracts prior to chromatographic separation. The pigments seemed bound to the ammonium sulfate crystals and, despite much agitation, incomplete re-entry into solution occurred. After it was found that such extracts were much more promptly and completely redissolved if the ammonium sulfate was eliminated from the preliminary protein precipitation, this modification was employed for most of the subsequent studies on hepatectomized dogs. In some instances, the ammonium sulfate was reduced in quantity but not deleted. The chromatographic column devised by Billing (20) was employed in the present studies.

The pigment fractions were measured by placing the pigment-containing segments of the column in separate, stoppered glass tubes. Five ml. of an alcoholic diazo solution was then added to each tube and the tubes were shaken thoroughly. The diazo solution was prepared as follows: Solution A: 1 Gm. of sulfanilic acid dissolved in 15 ml. of concentrated HCl, diluted to 1 L. with distilled water. Solution B: 0.5 Gm. of sodium nitrite ($NaNO_2$) in 100 ml. of distilled water. This solution was made daily before use. Mixed diazo reagent: 10 ml. of Solution A plus 0.3 ml. of Solution B. This reagent was used within 10 to 20 minutes after mixing. After standing with occasional further shaking for 25 minutes, the diazotized solution of pigment was removed from each tube by filtering through sintered glass filters, and the tubes and filters were washed with 5 to 10 ml. of absolute methanol to remove all traces of pigment. The diazotized solutions of pigment then were measured in cuvettes at 540 mμ with the Coleman Universal Spectrophotometer, Model 14. Calculation of the amount of pigment was made from the same calibration curve as that employed for the measurement of serum bilirubin, the values being corrected for a final volume of 9 ml., which was the volume used for measurement of total bilirubin in the serum bilirubin method employed. In presenting our results, Pigments I and II are expressed as percentages of the total direct-reacting bilirubin which additively they represent.

The method is at best difficult and time consuming. The chief technical difficulties are concerned with packing of the column and the achievement of clean-cut separation of the three pigments on the column. Experience and extreme care were needed to overcome these problems. All of the determinations in this study were performed by two of us (H. N. H. and F. F. W.).

The reproducibility of our results is comparable to that cited by Billing (20) and by Baikie (21). The concentration of bilirubin did not influence the reproducibility provided that the concentration of total bilirubin in the sample was at least 3 mg. per 100 ml. Recovery studies indicated that 90 per cent of the pigments in the protein-free extract were recovered from fractionation on the column whereas approximately 60 to 70 per cent of the original serum pigment was ultimately recovered after column separation. The losses of Pigments I and II during this procedure are apparently equal (20). Since our estimations have been concerned with the relative proportions of Pigments I and II and not their absolute values, the losses during protein precipitation and column separation do not influence our results.

Determinations of serum bilirubin were carried out on all serum tested. A modification of the Malloy-Evelyn method was used, with 1 minute and 15 minute direct readings and measurement of total bilirubin after the addition of alcohol (22).

Solutions of bilirubin for injection were prepared as follows: for dogs, 150 mg. of crystalline bilirubin was dissolved in 25 ml. of distilled water and 2 ml. of 0.1 N NaOH; for rats, 10 mg. of bilirubin was dissolved in 1.0 ml. of normal saline solution and 1.0 ml. of 0.1 N NaOH. The total volume was injected intravenously in bile fistula studies, and half the volume was administered to dehepatized rats. Suspensions of hemoglobin were prepared as follows: 15 Gm. of hemoglobin (from fresh dog erythrocytes hemolyzed in distilled water) was suspended in 120 ml. of normal saline solution for intravenous use in dogs. A similar preparation of rat hemoglobin was given to rats for which the usual dose was 300 mg. in 2 ml. of normal saline solution.

For the dehepatized dogs, solutions of bilirubin were injected intravenously within two hours of operation. Injections of hemoglobin (one to three 10 ml. aliquots) were given either 30 minutes prior to hepatectomy or within two hours after removal of the liver. The chromatographic determinations of serum pigment were all carried out at the termination of the experiments, which varied from 12 to 40 hours after injection.

The number of animals and patients studied and any special procedures taken to prepare them and to obtain test material will be described with the results.

RESULTS

Composition of bile

Bile obtained at operation from four human gall bladders and fistula bile from seven dogs and four rats were studied chromatographically. In all instances, most of the pigment in the bile was Pigment II. In human bile this fraction represented a mean of 73.1 per cent of the total, whereas in the bile of the dog and the rat, Pigment II was 75.1 and 84.8 per cent of the total, respectively (Table I).

Dehepatized dogs

Fourteen dogs were dehepatized after the method of Grindlay and Mann (23). They were maintained postoperatively by a saline-glucose solution administered by a constant injection apparatus. Ten dogs were given injections of hemoglobin or bilirubin. Two of the 14 dogs also underwent bilateral nephrectomy. Only dogs surviving 14 or more hours after operation were included in the present series.

Chromatography using the butanol pH 6 system invariably disclosed the presence of indirect-reacting bilirubin and one direct-reacting pigment. The latter was the dominant pigment in most instances. Further studies of the direct-reacting pigment fraction revealed that its mobility was somewhat less than that of known fractions of Pigment II, and that diazotization produced Pigment A and B bands, other characteristics of Pigment I (12). Additional chemical studies, that is, alkaline or enzymatic hydrolysis of Pigments A and B, were not carried out. The two hepatectomized, nephrectomized dogs yielded pigment ratios similar to those of the other animals, indicating that renal tissue was not responsible for the conjugation of bilirubin in the absence of the liver. Pigment patterns were not appreciably altered by injections of hemoglobin or bilirubin (Figure 1). The pigment in urine also was shown to be Pigment I.

Dehepatized rats

By a similar method 10 white Sprague-Dawley rats were hepatectomized. Of these, three were also eviscerated, and five, in addition to hepatectomy, underwent evisceration and nephrectomy. Injections of bilirubin were given to four of the animals after operation. All were maintained by continuous infusions of glucose in saline solution in the tail veins and were sacrificed approximately 24 hours after operation.

Indirect-reacting bilirubin, Pigment I and small amounts of Pigment II were present in the serum of all rats with intact kidneys. In contrast, Pigment II was absent in all of the nephrectomized

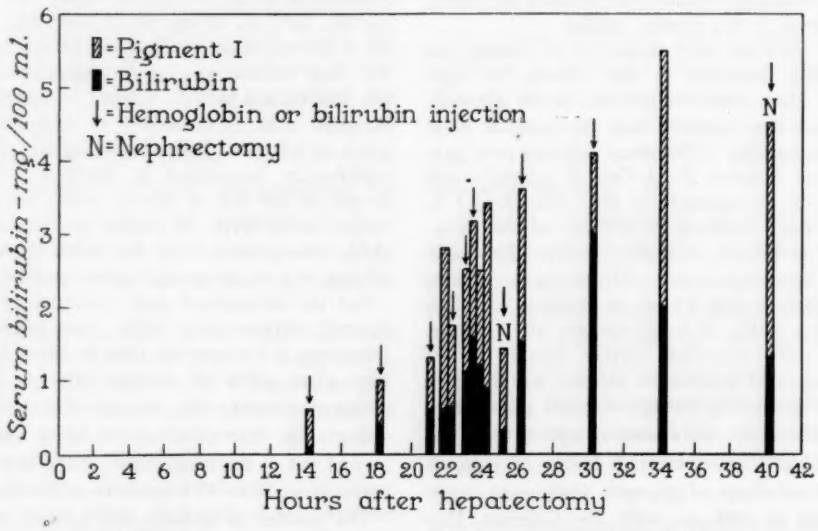
▨=Pigment I
▮=Bilirubin
↓=Hemoglobin or bilirubin injection
N=Nephrectomy
Serum bilirubin - mg./100 ml.
Hours after hepatectomy
0 1 2 3 4 5 6
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
N
N

FIG. 1. BILE PIGMENTS IN SERUM OF 14 DOGS AFTER TOTAL HEPATECTOMY
The word bilirubin is used to indicate the indirect-reacting bilirubin.

TABLE I

*Composition of pigment in bile**

Type	Pigment II	Pigment I
	%	%
Human		
1	70.5	29.5
2	72.0	28.0
3	86.0	14.0
4	64.0	36.0
Mean	73.1	26.9
Dog		
1	70.0	30.0
2	75.0	25.0
3	76.0	24.0
4	79.0	21.0
5	70.5	29.5
6	76.4	23.6
7	79.0	21.0
Mean	75.1	24.9
Rat		
1	90.3	9.7
2	80.0	20.0
3	80.0	20.0
4	89.0	11.0
Mean	84.8	15.2

* Pigments I and II are expressed as per cent of total pigment content.

animals. Indirect-reacting bilirubin was the predominant pigment in all except one of the preparations. As in the dehepatized dogs, pigment patterns were not appreciably altered by postoperative injections of bilirubin (Figure 2).

Bile following injections of hemoglobin and bilirubin

Biliary fistulas were surgically created by cannulation of the common bile duct following cholecystectomy in three dogs. The bile was collected over ice and protected from the light. It was studied chromatographically before and after injections of bilirubin and hemoglobin. Six white Sprague-Dawley rats were subjected to the same procedure.

All animals showed a two- to sixfold rise in total excretion of bile pigment following injections of bilirubin or hemoglobin. A twofold increase was present in all dogs and a sixfold increase in three of the six rats. There was a three- to tenfold rise in concentration of Pigment I, and a one- to sevenfold rise in concentration of Pigment II. Bilirubin was absent in all the dogs used as controls and present in traces in all except one of the rats used as controls. After the injections, there was a four- to twelvefold rise in excretion of bilirubin in five of the rats, and some bilirubin appeared in the bile of all dogs. Control levels appeared in approximately 48 to 72 hours in all of the animals. The overall results following injection of bilirubin or hemoglobin were similar in all respects. The bile-pigment pattern with respect to Pigments I and II remained normal in all of the rats following the injections but was variable in the three dogs.

Biliary obstruction in dogs

Twenty-two dogs were utilized in a study of the composition of bile pigment of peripheral blood following ligation of the common bile duct and cholecystectomy. In some animals hepatic lymph obtained by cannulation of the hepatic lymph vessels was studied.

In the first 72 hours after obstruction, there was a prompt elevation of direct-reacting pigment in the blood and hepatic lymph with a predominance of Pigment II (56 to 84 per cent of the total). This predominance, though in lesser degree, persisted for the first three to five weeks.

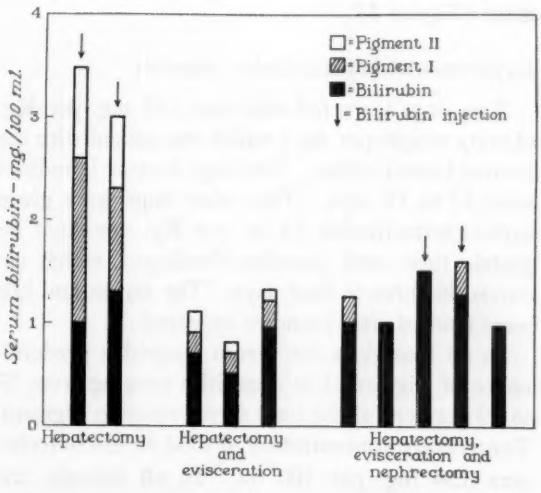

FIG. 2. BILE PIGMENTS IN THE SERUM OF 10 RATS AFTER TOTAL HEPATECTOMY ALONE OR WITH EVISCERATION OR WITH EVISCERATION AND BILATERAL NEPHRECTOMY

Bilirubin as used in this picture is the indirect-reacting type.

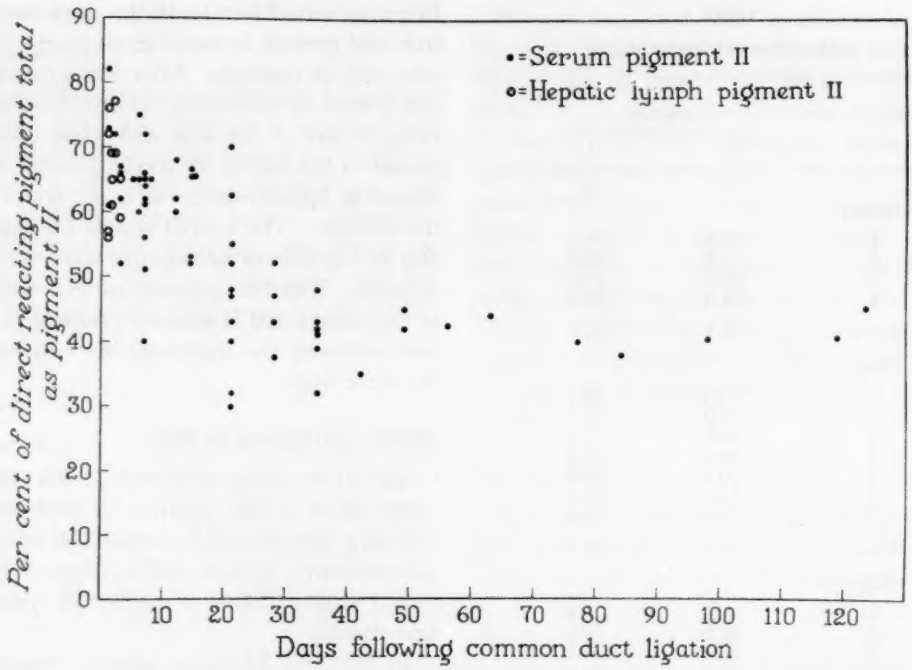

FIG. 3. DIRECT-REACTING BILE PIGMENTS IN SERUM AND HEPATIC LYMPH OF DOGS AFTER LIGATION OF THE COMMON BILE DUCT

Thereafter, measurements revealed that Pigment I was the chief pigment in serum, ranging from 56.5 to 68 per cent of the total direct-reacting pigment (Figure 3).

Experimental hepatocellular jaundice

Two dogs were fed ethionine (15 mg. per Kg. of body weight per day) which was mixed with the normal kennel ration. The dogs became jaundiced after 12 to 15 days. Two other dogs were given carbon tetrachloride (1 cc. per Kg. per day) by gastric tube until jaundice developed, which occurred in three to four days. The serum and bile were studied after jaundice appeared.

In all four dogs the serum showed a predominance of Pigment I in quantities ranging from 57 to 69 per cent of the total direct-reacting pigment. The average concentration of total serum bilirubin was 3.34 mg. per 100 ml. In all animals, the chromatographic pattern of the bile was normal.

Hepatocellular and obstructive jaundice

Serum from 147 jaundiced patients was studied. These patients were chosen almost solely by the criterion of a value for direct-reacting bilirubin of at least 3 to 4 mg. per 100 ml. of serum. In most of the cases, the diagnosis was unknown to us at the time the tests were done. Bile from 12 patients with hepatocellular jaundice who had elevated values for Pigment I in the serum was studied chromatographically. Eight patients had hepatitis and four had cirrhosis. Samples of bile were obtained by duodenal drainage in all except two cases in which it was obtained at necropsy by aspiration of the gall bladder.

Included in the 147 cases were 91 cases of extrahepatic obstructive jaundice. The etiologic factors in these 91 cases included stone or stricture of the common duct, carcinoma of the bile ducts, ampullary carcinoma and carcinoma of the pancreas. All diagnoses were substantiated at either operation or necropsy. In 80 of the 91 cases (87.9 per cent), Pigment II was the predominant pigment (Figures 4 and 5).

Fifty-six cases of hepatocellular jaundice were included in the group studied. Of these, 29 were cases of acute or subacute hepatitis and the remainder represented chronic hepatic disease,

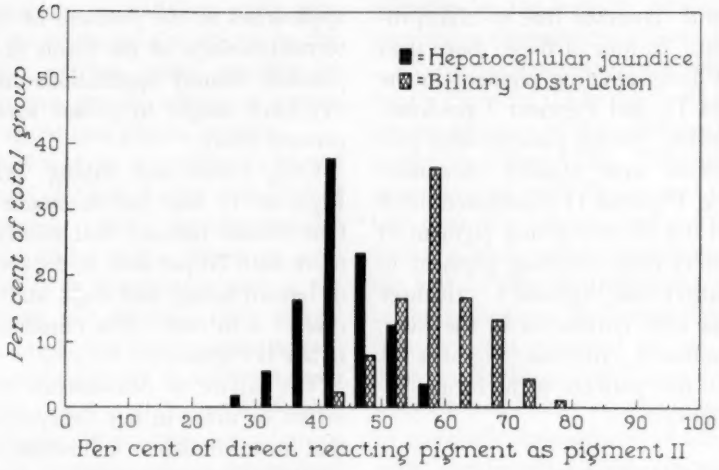

FIG. 4. PERCENTAGE OF PIGMENT II IN THE DIRECT-REACTING BILE PIGMENTS IN THE SERUM OF 147 PATIENTS WITH HEPATOCELLULAR AND EXTRAHEPATIC OBSTRUCTIVE JAUNDICE

namely cirrhosis of the portal and postnecrotic types. In 47 of these 56 cases (83.9 per cent), Pigment I represented more than 50 per cent of the total direct-reacting pigment (Figure 4).

Normal bile patterns were observed in all of the 12 patients with hepatocellular jaundice whose bile was studied chromatographically; this group included two patients who died in hepatic coma.

Other types of jaundice

A small number of patients with various other types of jaundice were studied.

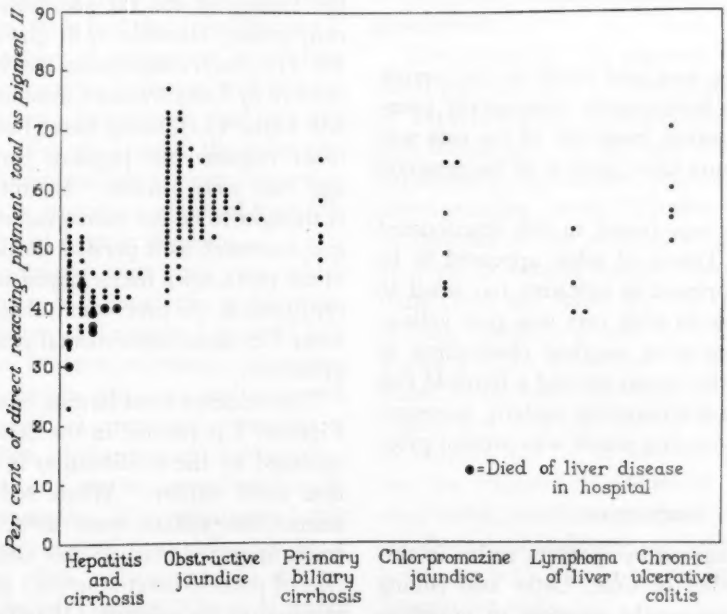

FIG. 5. DIRECT-REACTING BILE PIGMENTS IN SERUM OF PATIENTS WITH VARIOUS TYPES OF JAUNDICE

Eight patients with jaundice due to chlorpromazine were studied. In four of these, more than 50 per cent of the direct-reacting pigment in the serum was Pigment II, and Pigment I predominated in the remainder. Seven patients with primary biliary cirrhosis were studied chromatographically. In five, Pigment II constituted more than 50 per cent of the direct-reacting pigment in the serum. The chief direct-reacting pigment in the serum of the others was Pigment I. In three of the four patients with lymphoma in the liver, Pigment I predominated, whereas Pigment II was dominant in all five patients with chronic ulcerative colitis (Figure 5).

Constitutional hepatic dysfunction

Chromatographic studies were carried out on the serum from eight patients with constitutional hepatic dysfunction. The bile from five of these was studied also, and determinations of fecal urobilinogen were made on three of the patients whose bile was studied.

Bilirubin was the only pigment in the serum in each case. In those patients whose bile was studied, a normal pigment pattern was observed. Values for fecal urobilinogen were normal in the three cases.

The Gunn rat

Chromatography was performed on the serum and bile from two homozygous congenitally jaundiced rats. The serum from one of the rats was fractionated 24 hours after ligation of the common duct.

Bilirubin alone was found in the fractionated serum and bile. Traces of what appeared to be Pigment I were present in amounts too small to measure. The bile in both rats was pale yellow. Twenty-four hours after surgical obstruction in one of these rats, the serum showed a fourfold rise in bilirubin. The fractionation pattern, however, was unchanged from that which was present prior to obstruction.

DISCUSSION

It is generally agreed by workers active in this field that the studies of Cole, Lathe and Billing have provided long-sought answers to questions concerning the nature and behavior of bilirubin. At the same time, new questions as well as new approaches to the problem of the physiologic interrelationships of the forms of bilirubin and the possible clinical applications have been created. We have sought to pursue some of these in the present study.

Cole, Lathe and Billing (11) observed that Pigment II was the dominant pigment in bile. Our studies indicate that this fraction constitutes more than 70 per cent of the total pigment in bile of human beings and dogs, and more than 80 per cent of it in rats. The remainder of the pigment in bile is Pigment I.

The failure to demonstrate Pigment II in the serum or urine in our dehepatized dogs indicates that its formation is a function of the liver. The presence and the progressive accumulation of direct-reacting Pigment I in addition to indirect (unconjugated) bilirubin in all our dehepatized dogs are not in accord with previous concepts of bilirubin metabolism which assign to the liver the role of transforming indirect bilirubin to direct bilirubin. Bilateral nephrectomy in two dogs at the time of hepatectomy did not appear to diminish the concentration of Pigment I. The continued presence of small amounts of Pigment II in dehepatized, eviscerated rats, however, with its disappearance after nephrectomy, suggests that the kidney of the rat possesses the capacity for conjugating bilirubin with glucuronic acid (Figure 2). Such conjugation has been demonstrated *in vitro* by Grodsky and Carbone (24), and Billing and Lathe (13), using tissue homogenates. In all other respects, the pigment patterns in the dogs and rats were similar. Inasmuch as Pigment I is thought to be the monoglucuronide of bilirubin, and inasmuch as it persists despite the elimination of the two known major organ sites of glucuronide conjugation, the precise extrahepatic origin of Pigment I in these experimental preparations remains unknown.

The evidence cited leading to the conclusion that Pigment I is formed in the dehepatized animal is qualified by the modification in protein precipitation cited earlier. When reduced amounts of ammonium sulfate were used, the pigment extracts appeared more soluble and a greater proportion of polar (direct-reacting) pigment was noted moving on the column. If fully prescribed quantities of ammonium sulfate were used, however, little or no polar pigment was recovered from the

column. These variations in yield of polar pigment that seemed to relate to alterations in the protein precipitation process could possibly be explained by the known greater affinity of conjugated bilirubin for denatured plasma protein.

It might be argued that, in accordance with the extraction procedure of Cole, Lathe and Billing (11), no direct-reacting (polar) pigment was detectable in the dehepatized dog. However, certain observations make us certain that such is not the case: *1*) Bollman and Mendez (25) clearly demonstrated that, employing the chloroform, carbon tetrachloride, methanol, pH 6 solvent system which separates the direct and indirect-bilirubin fractions, a significant direct-reacting pigment fraction was present in the serum of dehepatized animals, and we have confirmed this many times. *2*) By the van den Bergh test, direct-reacting pigment (1 minute fraction) was always demonstrable in the serum of such animals, the quantity varying from 10 to 50 per cent of the total measurement. *3*) For many years direct-reacting bilirubin has been found in the urine after total hepatectomy (3). *4*) In studies on the dehepatized rat, full amounts of ammonium sulfate were used, and Pigments I and II were present in all columns. These observations, we think, are strongly suggestive that polar (conjugated) pigment is formed in the absence of the liver and that it is Pigment I. The amount and nature of conjugates which may be present in this fraction, however, will require isolation and analysis, procedures that are not presently possible. For the present, the polar pigment of the hepatectomized dog appears chromatographically similar to Pigment I obtained from bile or from icteric sera of dogs or humans.

In the hepatectomized dogs and rats which were given injections of hemoglobin or bilirubin, the relative proportion of Pigment I to total bilirubin in the serum was not increased. However, injections of hemoglobin into dogs and rats with biliary fistulas resulted in increased excretion of Pigment I in the bile. This seems to indicate that at least some Pigment I can be formed within the liver. From these studies, it is hypothesized that Pigment II may be formed from bilirubin in at least two different sites. These are: *1*) the extrahepatic formation of Pigment I from bilirubin and its subsequent conversion within the liver to Pigment II; and *2*) the direct conjugation of bilirubin by the liver to form Pigment I and its conversion thereafter to Pigment II.

The prompt appearance of pigments (predominantly Pigment II) in the blood in our animals following ligation of the common duct has been reported by Billing (26). This might be anticipated in accordance with the concept of regurgitation of bile from the biliary system into the circulation. Billing's observations, however, were confined to the first 12 days of obstruction, and no suggestion of the subsequent decline in the Pigment II fraction that was apparent in our series was noted. The predominance of Pigment I in our animals with chronic biliary obstruction may be explained by metabolic derangement of the parenchymal cells with impairment of the glucuronide-conjugating mechanism which resulted from the prolonged total obstruction.

In contrast to the greater accumulation of Pigment II following experimentally produced biliary obstruction, the jaundice in dogs which followed hepatic injury by carbon tetrachloride or ethionine was characterized by a predominance of Pigment I. This finding we have interpreted as suggesting strongly that jaundice due to damage of the parenchymal cells is due to impaired conjugation and excretion of most of the bilirubin as the diglucuronide, with resultant accumulation of the monoglucuronide.

The values for the pigment fractions in jaundiced patients were consistent with those obtained in experimentally produced jaundice in animals just discussed. In 87.9 per cent of the cases of extrahepatic obstruction, the major portion of the direct-reacting serum bilirubin was Pigment II. In contrast to the obstructive jaundice produced in dogs, prolonged human obstruction, which in some cases had lasted for more than six months, did not result in a loss of the predominance of Pigment II in the serum. The human liver appears to be more resistant to prolonged biliary obstruction than our earlier observations seemed to indicate (27). It was not possible to correlate the Pigment II values in the patients with obstructive jaundice with levels of serum bilirubin or alkaline phosphatase, or with the thymol turbidity, or with results of the cephalin-cholesterol flocculation tests.

Of the group with hepatocellular jaundice, 83.9 per cent showed a predominance of Pigment I in

the direct-reacting serum bilirubin. As in the group with obstructive jaundice, there was no positive correlation between the results of any of the conventional tests of liver function and the pigment values. However, the lowest values for Pigment II were obtained in the patients most severely ill with hepatitis and cirrhosis. Hospital deaths from liver disease in our series occurred largely within this group. Hence, it might be concluded that a low Pigment II value in a patient with parenchymal jaundice is an indication of the severity of the disease process. In a number of these patients the clinical and laboratory features did not strongly suggest hepatocellular jaundice. Chromatography in all of these cases, however, showed definite predominance of Pigment I, and a diagnosis of hepatocellular jaundice was eventually substantiated.

In contrast to the greater amounts of Pigment I in the serum of patients with infectious hepatitis, the pigment pattern of bile obtained by duodenal drainage in 12 of these patients did not differ from that of normal bile. It is possible that a minimal number of intact liver cells is able to excrete normally conjugated bile pigments and that the damaged cells are not only unable to conjugate but are also incapable of excreting conjugated pigments. Less likely is the possibility that Pigment II is formed by the epithelial cells of the extrahepatic biliary tract. A final explanation of this phenomenon awaits further study.

In most of our patients with primary biliary cirrhosis and in half of those with chlorpromazine jaundice (both of these conditions are examples of intrahepatic obstruction), the pigment pattern was similar to that seen in extrahepatic obstruction. However, in two patients with primary biliary cirrhosis and in four with chlorpromazine jaundice, Pigment I was dominant. Although our series is small, it indicates that in some cases pigment partition may be of diagnostic aid in demonstrating hepatocellular damage associated with intrahepatic obstruction and in differentiating it from extrahepatic obstructive jaundice.

The elevation of Pigment I in all but one of our patients with lymphomatous involvement of the liver is compatible with the presence of diffuse cellular dysfunction. In contrast, an obstructive pattern was present in all patients with jaundice secondary to chronic ulcerative colitis.

The results discussed above concerning obstructive and hepatocellular jaundice differ from those reported in the recent studies of Billing (26) and of Baikie (21). In 14 cases of obstructive jaundice and three of acute hepatitis, Billing observed that Pigment I in all instances was dominant. Baikie observed no consistent pattern of diagnostic value in 46 determinations in 12 patients with hepatocellular disease and two patients with extrahepatic biliary obstruction. Careful scrutiny of these data, however, shows that Pigment II was dominant in the majority of determinations in the group with extrahepatic biliary obstruction whereas Pigment I was more commonly elevated among the patients with parenchymal jaundice. It is difficult to explain the differences in our figures and those of Billing despite personal discussion of our respective results. It is our opinion that estimation of the serum bile pigment fractions offers a potential means of differentiating parenchymal from obstructive jaundice. However, the reproducibility and the technical difficulties inherent in the method limit its diagnostic value in its present form.

Recent studies have clarified the mechanisms involved in the conjugation of bilirubin by the liver. These have shown that glucuronic acid is transferred to bilirubin from uridine diphosphate glucuronic acid in a reaction catalyzed by the enzyme, glucuronyl transferase, which is present in the microsomes of the liver (24, 28, 29). Using liver homogenates, Carbone and Grodsky (30), Schmid, Hammaker and Axelrod (28), and Lathe and Walker (29) have demonstrated marked impairment in the conjugation of bilirubin in rats with congenital nonhemolytic jaundice due to a deficiency or absence of this enzyme system. The virtual absence of direct pigment in the colorless bile of similar rats in the present study is consistent with such a defect.

In our eight patients with constitutional hepatic dysfunction, no conjugated pigment was found in the serum. The normal composition of bile in these patients was intriguing, since Arias and London (31) have shown deficient glucuronyl transferase activity in the liver of two patients with this disease. It is of interest, however, that bile aspirated from the gall bladder of one of their patients gave a direct van den Bergh reaction. Bile from the other patient was not studied. In

addition, a similar defect in transferase activity was demonstrated in the three children with congenital nonhemolytic nonobstructive jaundice studied by Axelrod, Schmid and Hammaker (32). The bile, however, was colorless and contained only traces of unconjugated bilirubin. Serum bilirubin levels were much higher in these patients than in ours.

Since ratios of conjugated bilirubin in bile and values for fecal urobilinogen were normal in our cases of constitutional hepatic dysfunction, it is difficult to postulate a defect in the mechanism of excretion of bile pigment. Inasmuch as constitutional hepatic dysfunction is a nonspecific disease entity (33), our patients may not be identical to those described by the other authors. Quantitative differences in glucuronyl transferase activity or the presence of other conjugates of bilirubin may explain these differences.

SUMMARY AND CONCLUSIONS

A study of bile pigments employing the method of reverse phase partition chromatography has been presented. This has included a variety of studies on animals intended to define more clearly the metabolic pathways and interrelationships of the pigments, and an analysis of the pigment patterns observed in the serum and bile of humans and animals with various types of jaundice. The following conclusions have been drawn.

1. The pigment in bile of normal humans, dogs and rats is 75 to 80 per cent Pigment II, the remainder being Pigment I.

2. Pigment I and free bilirubin appear in the serum of the dog and the rat after total hepatectomy. Since essentially no Pigment II is formed in the absence of the liver, and as it is the dominant pigment in bile, it is concluded that the liver is the major site of formation of Pigment II.

3. In addition to the extrahepatic formation of Pigment I and its subsequent conversion by the liver to Pigment II, our studies suggest that some Pigment I is formed from bilirubin in the liver and converted thereafter to Pigment II.

4. Experimental biliary obstruction in animals is characterized initially by a predominance of Pigment II in the serum, a pattern reflecting that of normal bile. However, in animals with induced hepatocellular jaundice, Pigment I is the chief serum pigment. This strongly suggests that damage to liver cells impairs the conjugation and excretion of the majority of the bilirubin as Pigment II, with the resultant accumulation of Pigment I.

The last hypothesis is strongly supported by a clinical study of jaundice in humans. Pigment II was the dominant direct-reacting pigment in the serum of 87.9 per cent of the patients with extrahepatic obstructive jaundice. Conversely, Pigment I was the chief pigment in the serum of 83.9 per cent of the patients with hepatocellular disease. Of interest is the observation that the pigment ratio in the bile of these patients was normal. The possible diagnostic and prognostic application of this approach to the clinical evaluation of jaundice has been discussed. In our opinion, the separation and measurement of serum bile pigment fractions is a potentially satisfactory method for the differentiation of parenchymal and obstructive jaundice. However, the technical difficulties inherent in the current method both limit its diagnostic accuracy and make its general use impractical at present.

5. Our studies confirm the presence of a defect in the conjugation of bilirubin in rats (Gunn strain) with congenital hyperbilirubinemia.

6. The results of our studies on patients with constitutional hepatic dysfunction do not support the concept of absent or defective glucuronyl transferase activity. Other possible interpretations of our results have been discussed.

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EFFECT OF DEEP AND QUIET BREATHING ON PULMONARY COMPLIANCE IN MAN * †

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Observations on convalescent patients with respiratory muscle paralysis due to poliomyelitis have shown that their lungs are approximately half as compliant as normal (1). A similar reduction in pulmonary compliance has been observed in patients under anesthesia (2). Other workers have reported that during the acute stages of poliomyelitis the compliance of the lungs and thorax decreased as the vital capacity decreased (3). Reduction in pulmonary compliance has been observed in anesthetized dogs and on excised dogs' lungs; such changes could be prevented if the lungs were occasionally stretched by means of a deep breath (4). The use of occasional deep breaths was recommended in one of the earliest reports on the treatment of patients with respiratory muscle paralysis (5) and clinical observations on the vital capacity of patients with moderate residual respiratory muscle weakness due to poliomyelitis have shown that the vital capacity is increased by a series of assisted maximal inspirations (6). Since the fall in compliance in patients with poliomyelitis has been observed to parallel the fall in vital capacity (3, 6) the question may be asked whether similar reductions in lung compliance occur in intact unanesthetized human beings when they breathe quietly. If similar changes occur it would be of interest to determine their magnitude, how quickly they develop and whether they could be reversed. It would also be pertinent to determine whether the decreased compliance observed in poliomyelitic patients could be increased. Such information would be useful to guide the treatment of patients who require prolonged artificial respiration, so that reductions in compliance might be minimized.

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METHODS

Measurements were made on 11 adult convalescent poliomyelitic patients and 10 normal adults. The patients used a tank respirator since they had markedly reduced breathing capacities. The patients were studied from six months to three years after the onset of their acute illness.

Change in esophageal pressure obtained from a latex balloon approximately 15 cm. in length and with the tip probably in the lower third [1] of the thoracic esophagus was used as an index of change in pleural pressure (7). The pressure difference between the esophageal balloon and mouthpiece was measured by an inductance manometer, and was used to represent transpulmonary pressure. In most of the studies involving the patients, the pressure difference from the tank to the mouth was also measured and represented the pressure differences across the lungs and thorax.[2] Volume changes were obtained from a closed circuit recording spirometer (modified Benedict-Roth) and recorded electrically by means of a rotating potentiometer. Airflow was measured by means of a pneumotachograph. Pressure, volume and flow changes were electrically amplified and recorded simultaneously on a multi-channel, direct-writing oscillograph.

Pulmonary compliance was calculated as the ratio of change in volume to change in "transpulmonary" pressure at points of zero airflow during quiet breathing. An average value determined from at least 10 respiratory cycles was used to represent the lung compliance. In a similar manner the compliance of the lungs and "thorax" was measured in the patients, and the "thoracic" compliance was calculated. Respiratory frequencies ranged from 9 to 18 per minute, with the lower frequencies in the normal subjects.

It was recognized that the esophagus as a site for pressure recording was subject to considerable error especially in the supine position (8). Since direct intrapleural pressure measurements were not practical, the inherent variability of the esophageal site had to be accepted as a part of the variations in the data collected. To minimize

[1] The balloon on the end of a polyethylene catheter was passed through the nose. The distance from the tip of the balloon to the nares ranged from 35.5 to 45.5 cm., depending upon the size of the subject.

[2] Thorax here is used to include rib cage, diaphragm, and abdominal wall and abdominal contents.

TABLE I

Patients

Patient	Sex	Age	Ht.	Wt.	Supine vital capacity	Supine functional residual capacity	supine residual volume	Continuous unassisted time per day
		yrs.	*cm.*	*Kg.*	*L.*	*L.*	*L.*	
E. C.	M	31	183	82.0	0.20	2.21	2.21	1–2 min.
J. G.	F	23	160	43.0	0.35	1.80	1.80	4–5 min.
P. G.	F	21	157	29.0	0.58	2.59	2.59	8 hrs.
M. H.	F	30	167	39.5	0.27	1.34	1.34	½ hr.
N. H.	M	19	183	53.0	1.10	2.51	2.40	10–12 hrs.
H. J.	M	41	177	66.0	0.40	2.62	2.62	without GPB 1 hr.*
M. M.	F	40	160	42.0	0.20	1.97	1.97	1–2 min.
E. R.	F	29	162	51.0	0.22	2.03	2.03	1–2 min.
M. S.	F	17	170	33.5	0.55	1.62	1.54	2–3 hrs.
R. T.	M	38	182	62.0	0.33	2.44	2.44	5–10 min.
W. W.	M	16	180	44.0	0.24	1.60	1.60	5–6 min.

* GPB = glossopharyngeal breathing. With this type of respiration H. J. could inflate his lungs to a volume of 1.90 L. using 16 strokes. His continuous unassisted time could then be extended to six to seven hours.

variation in pressure measurements due to heart beats, a mean of the cardiac oscillations was estimated.

Measurements were made on the patients and subjects in the supine, lateral and prone positions. To examine whether changes occurred in the sitting position, the pulmonary compliance in the tidal volume range was measured at two lung volumes; one was the supine and the other the sitting functional residual capacity.

This was accomplished in the following manner: the supine resting end-expiratory position was monitored on a recording spirometer prior to position change and the oxygen consumption was approximately balanced by adding oxygen. Position change was accomplished by means of a tilting Gatch bed accompanied by appropriate adjustments to make a comfortable "chair." Any change in the resting end-expiratory position due to position change was recorded on the spirometer. To maintain a constant lung volume from the supine to the sitting position, an abdominal binder with inflatable balloon was used with pressure maintained while the subject was in the sitting position. No change in the resting end-expiratory position was recorded when the balloon was inflated beneath the binder as the subject was tilted into a sitting position.

When lung volume change was permitted to occur, no binder was used and gravity produced the lung volume change in the relaxed subject. The functional residual capacity (FRC) was measured in the supine position by means of the helium dilution method (9). Supine vital capacities and expiratory reserve volumes were obtained on the recording spirometer. All values were corrected to body temperature and pressure and saturated with water vapor (BTPS).

Deep breaths were achieved for the patients by increasing the negative tank pressures to 30 to 35 cm. H_2O. Peak pressures were held for two to three seconds. The normal subjects inhaled spontaneously to their inspiratory limit. Pulmonary compliance was measured after completing a series of four maximally deep breaths, or after one or two deep breaths during the series. The purpose of the latter procedure was to determine whether there was a progressive change in compliance after each deep breath or whether one or two deep breaths produced most of the change. Compliance measurements were also made after a series of small, moderate and large increases in lung volume to observe whether or not there was a progressive increase in pulmonary compliance associated with progressively deeper breaths.

Following a series of deep breaths, quiet breathing was resumed. Patients breathed at their usual tank pressure and frequencies. The normal subjects breathed at a relatively constant and natural tidal volume. Esophageal pressures were monitored during the rest period to ensure that no deep breaths had been taken. The pulmonary compliance in the tidal volume range above the FRC was measured at intervals during the periods of quiet breathing.

TABLE II

Normal subjects

Subject	Sex	Age	Ht.	Wt.	Supine vital capacity	Supine functional residual capacity	Supine residual volume
		yrs.	*cm.*	*Kg.*	*L.*	*L.*	*L.*
D. C.	M	28	184	82.0	5.52	2.39	1.54
R. D.	M	41	196	81.6	6.33	3.90	2.23
B. F.	M	38	188	82.0	5.80	3.36	2.12
R. F.	M	33	178	82.0	4.48	3.26	2.30
S. H.	F	33	155	54.5	3.05	1.79	1.28
A. J.	M	32	181	71.5	5.42	2.94	1.97
J. M.	M	37	187	86.5	6.00	4.00	2.63
D. P.	M	33	185	66.0	5.81	3.45	2.40
M. T.	F	64	162	62.0	2.30	2.16	1.75
N. W.	F	33	170	55.0	4.54	3.86	2.51

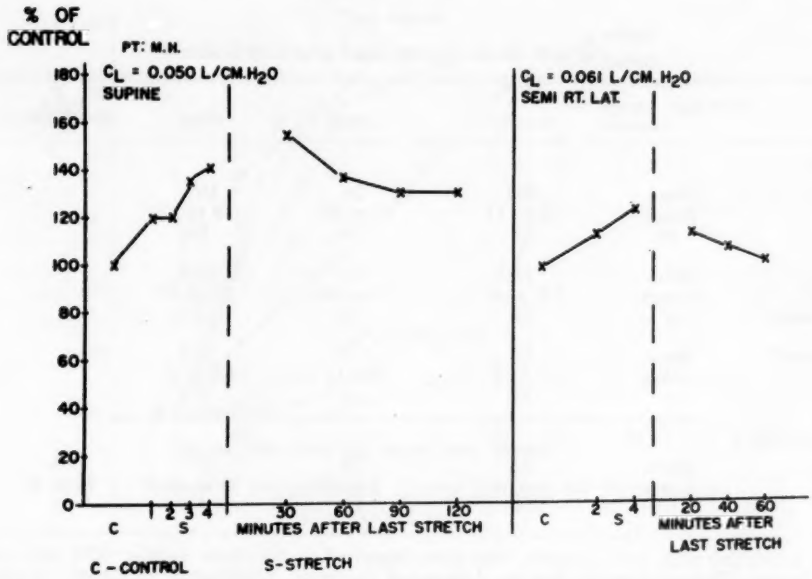

FIG. 1. PER CENT CHANGE IN COMPLIANCE OF LUNGS AS RESULT OF DEEP AND SHALLOW BREATHING

RESULTS

The physical characteristics, vital capacity and spontaneous unassisted time of the patients are presented in Table I, and those of the subjects in Table II.

Typical results obtained with a patient in supine and semi-right lateral position are presented in Figure 1; similar data obtained on a normal subject are shown in Figure 2. In both studies there was a marked increase in the pulmonary compliance as measured in the tidal volume range following deep breaths. In the normal subjects this change appeared to be complete after two deep breaths, whereas in the patients there was a progressive rise. During the period of quiet breathing both showed a fall of the compliance toward the control values, which was faster in the normal subjects than in the patients. The increase in compliance could be reproduced when another series of deep breaths was inhaled as shown in the subject in Figure 2.

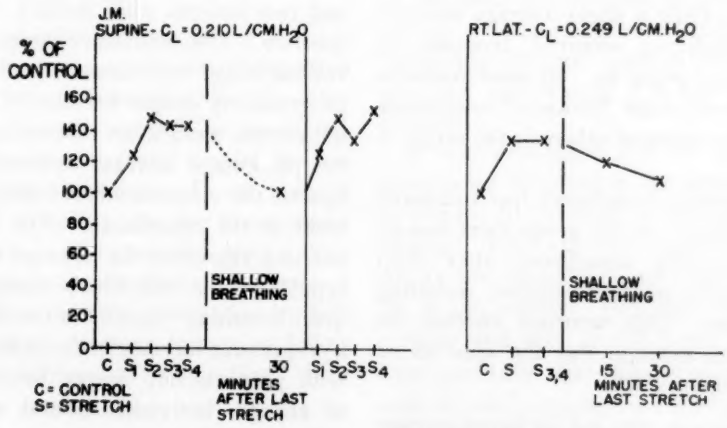

FIG. 2. CHANGE IN COMPLIANCE OF LUNGS AS RESULT OF DEEP AND SHALLOW BREATHING

TABLE III *

Per cent change in compliance after deep breaths

		Supine	Lateral	Prone	Sitting (upright FRC)	Sitting (supine FRC)
Patients						
Lungs	Mean	29	26	40		
	Range	12 to 54	11 to 49	28 to 52		
	n†	23	8	2		
Lungs and "thorax"	Mean	17	13	22.5		
	Range	2.5 to 36.5	11 to 14	15 to 30		
	n	23	4	2		
"Thorax"	Mean	21	2	6.2		
	Range	−12 to 83	−25.5 to 18	4.5 to 8		
	n	23	4	2		
Normal subjects						
Lungs	Mean	32	29	29	36	20
	Range	5 to 51	5 to 49	16 to 38	26 to 45	7 to 42
	n	9	10	4	3	5

* Data on which this table was compiled have been deposited as document number 6070 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. Enclose remittance of $1.25 per copy for a photoprint or a microfilm.

† n Represents the number of subjects. Where multiple studies were done in the same position on the same person, the average was used.

The average percentage change in compliance after deep breaths in the various body positions is presented in Table III. For the patients this change is shown for the lungs, lungs plus "thorax," and "thorax" alone. In each of the three positions studied—supine, lateral,[3] and prone—there was an increase in the average pulmonary compliance. The range of increase obtained, however, was considerable. Similarly there was an increase in the compliance of the lungs plus "thorax." When the "thoracic" compliance was calculated the average increase in the supine position was similar to that seen in the lung. Only a slight average increase in "thoracic" compliance occurred, however, in the prone and lateral positions. In some instances there was a decrease in the "thoracic" compliance as indicated by the negative values in the range of observations.

Only the pulmonary compliance was measured in the normal subjects. In this group there was an increase in pulmonary compliance after deep breaths in each of the positions studied, including the sitting position. This occurred whether the FRC in the sitting position was the same as or larger than the supine FRC.

[3] Since measurements in right and left lateral positions were similar and lung volumes in these two positions are comparable (10), these data have been pooled.

The pulmonary compliances obtained in the normal subjects were considerably greater than those obtained in the patients in comparable body positions. The values for the subjects were 2.6 to 3.1 times greater than those obtained in the patients, even when differences in height were taken into account.

Since the foregoing results were obtained after maximally deep breaths it was of interest to determine whether this change occurred at a critical volume (or pressure) or whether it showed a gradual and progressive increase. Five subjects and two patients were studied to investigate this question. The pulmonary compliance in the tidal volume range was measured after each series of progressively deeper breaths. The results in the individuals studied are shown in Figure 3 where the percentage increase in compliance is plotted against the volumes inhaled prior to the measurement of the compliance. The intercepts on the abscissa represent the average tidal volumes accepted by the individuals during the period of quiet breathing. In all but one instance there was a progressive increase in the pulmonary compliance with progressively deeper breaths. The degree of change, however, showed considerable individual variation.

To determine how promptly the increase in

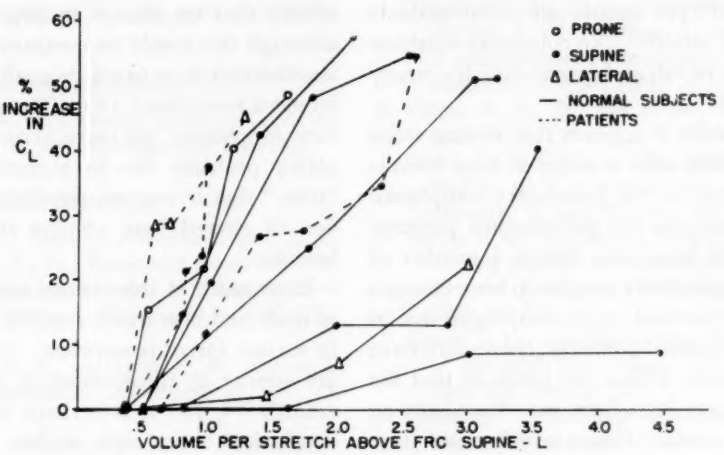

FIG. 3. EFFECT OF PROGRESSIVELY DEEPER BREATHS ON PULMONARY COMPLIANCE (C_L)

compliance could be reversed, forced expirations below the resting end-expiratory position were performed. This was done immediately after the measurement of compliance following a series of maximally deep inspirations. This maneuver was attempted in two of the patients, but the positive tank pressures obtained (10 to 15 cm. H_2O) were insufficient to produce a change in pulmonary compliance. In five normal subjects, however, spontaneous forced expirations to the residual volume produced a prompt and marked decrease in the pulmonary compliance and a return of the pulmonary compliance close to the lowest value previously obtained.

DISCUSSION

In the process of collecting the data it was apparent that there was considerable variability from day to day on the same individual. This amounted to 12 to 20 per cent in the "control" values of pulmonary compliance and occurred despite the use of essentially the same technique. It was thought that much of this variability was due to variations in esophageal "tone." For example, after a deep breath there was occasionally obvious evidence of esophageal activity which was manifested by failure of the esophageal pressure change to follow the lung volume changes. At other times there was a shift in the resting end-expiratory esophageal pressure either to a more negative or to a more positive value. In six instances, involving five normals and one patient, studies were rejected because of extreme variations of this type. Except for one normal subject all were repeat studies. Much difficulty was encountered in passing the esophageal balloon in the single study on the normal subject and there was obvious esophageal activity. The compliance values obtained in these rejected studies revealed four normal subjects with extremely high lung compliances (0.4 to 0.5 L. per cm. H_2O); deep breaths produced relative increases in lung compliance comparable to the values reported. In two other instances deep breaths produced essentially no change in pulmonary compliance. One of these was the single study on the normal subject. This was contrary to the usual observations seen in the other individuals. When all the rejected data were included, the average increase in the different body positions was not significantly different from the values reported.

It was anticipated that the more rapid fall in compliance observed in the normal subjects might be related to the tidal volumes or breathing frequency. Tidal volumes ranged from 500 to 1,200 ml. in the normal subjects and from 200 to 650 ml. in the patients. No relationship between tidal volume and rate of fall could be demonstrated, even when the tidal volume was expressed as per cent of vital capacity in the subjects or per cent of predicted vital capacity in the patients. Frequencies of breathing in the normal subjects

ranged from 9 to 16 per minute and in the patients from 14 to 18 per minute. No consistent relationship between rate of fall and respiratory frequency was shown.

From these results it appears that during quiet or shallow breathing after a series of deep breaths there was a decrease in the pulmonary compliance in both normal subjects and poliomyelitic patients. This change could have been due to a number of factors; for example, there may have been changes in the pressures recorded in the esophagus due to hysteresis of mediastinal contents; there may have been closure of units within the lungs so that the effective volume was less; there may have been an alteration of the surface forces which could have resulted in the closure of pulmonary units; or a change in surface forces might produce a change in pulmonary compliance without causing closure of pulmonary units.

This change in pulmonary compliance appears to be real and not due to hysteresis of mediastinal contents or other artifacts associated with using esophageal pressure as an index of intrapleural pressure. This is supported by the observations that similar changes in pulmonary compliance occurred in a variety of body positions in which artifacts resulting from using esophageal pressure would be minimized. Furthermore, changes were observed in the lungs plus "thorax" in patients in whom no esophageal pressures were used.

Whether there was closure of units cannot be decided from these data. The functional residual capacity was measured in the supine position before and after stretching in eight patients, but no significant difference was found between the two series. The time required to make the measurements was 15 to 20 minutes. This time period would have been sufficient for any increase in the FRC to have reverted in the normal subjects since they showed a fall in pulmonary compliance over a comparable interval of time. On the other hand, the patients did not show a prompt fall in compliance and a closure of pulmonary units would not seem to have occurred. The other and more likely probability is that the helium method used did not have sufficient sensitivity to detect a small difference in the FRC. Comparison of paired measurements of the functional residual capacity on supine patients and upright subjects has shown a variation of ± 6 per cent. There is also the possibility that no change in lung volume occurred, although this would be contrary to the findings on anesthetized dogs in which small but significant differences were found (4). The resting end-expiratory esophageal pressure showed so much variability probably due to alterations in esophageal "tone," that it was not possible to use it as an index of altered lung volume after maximal deep breaths.

How much of this change resulted from closure of units and how much resulted from an alteration in surface forces is not clear. Since these changes are similar to those noted in other experimental animals (4, 11) it is perhaps reasonable to apply conclusions from such studies to human beings. If this is done, then the changes in compliance would be explained on the basis of both phenomena, a closure of some units, and an alteration in surface forces in others. One might conjecture that sighing may be an expression of a reflex mechanism which attempts to correct these changes and make the lungs more compliant.

One can also conclude that when deep breaths produce an increase in pulmonary compliance they are beneficial since less energy will be required for breathing in the tidal volume range; and to the extent that there is a more uniform distribution of open alveoli there will be a better distribution of gas within the lungs. It is pertinent to point out that deep breathing should minimize the changes in pulmonary compliance associated with severe respiratory muscular weakness due to poliomyelitis provided it was done sufficiently often. Furthermore, spontaneous or assisted deep breaths during postoperative recovery might minimize the occurrence of atelectasis.

SUMMARY

Convalescent poliomyelitic patients and normal subjects showed a decrease in their pulmonary compliance as measured in the tidal volume range during quiet breathing after a series of deep breaths. This change occurred in the prone, supine, lateral and sitting positions. Compliance decreased 26 to 40 per cent. Two or more deep breaths to the limit of inspiration, after the period of quiet breathing, produced an increase in the compliance; this increase could be eliminated by forced expirations in the normal subjects. It was thought that these changes were probably due to

the opening and closing of various units within the lung.

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THE ESTERIFICATION OF PALMITATE-1-C^{14} BY HOMOGENATES OF INTESTINAL MUCOSA * †

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The esterification of long chain fatty acids has been shown to represent an important phase of fat absorption in the rat and in man (1–4). Although some glycerides are not completely hydrolyzed prior to absorption, a large proportion of the fatty acids is liberated in the intestinal lumen (5, 6) only to appear in the lymph as neutral fat in chylomicrons (7–9). In contrast, short chain fatty acids pass largely unchanged into the portal vein (3, 10, 11). The conversion of long chain fatty acids into neutral fat occurs in the mucosa of the small intestine, but the processes involved have not been clearly demonstrated. Favarger and Gerlach (12) supported the hypothesis that this formation of neutral fat was due to a synthetic action of a mucosal lipase. However, the demonstration in liver that the activation of a fatty acid by Coenzyme A (CoA) and adenosine triphosphate (ATP) is a necessary preliminary step prior to glyceride bond synthesis (13–17) has suggested that a similar mechanism may obtain in the mucosa of the small intestine.

In the present investigation, by using homogenates of rat and human intestinal mucosa, a very active system has been demonstrated which is capable of incorporating long chain fatty acids into neutral fat. This system is dependent upon ATP and CoA and is more active in the jejunum than in the ileum or colon. Short chain fatty acids are not incorporated. It has been observed that homogenates from the jejunum of patients with idiopathic steatorrhea possess greatly diminished activity.

MATERIALS AND METHODS

C^{14}-labeled fatty acids were obtained commercially (Volk Radio-chemical Company). Both palmitic acid-1-C^{14} and stearic acid-1-C^{14} had a specific activity of 5×10^6 cpm per μMole. The C^{14}-labeled palmitic acid contained 6 per cent of a neutral radioactive impurity while the C^{14}-stearic acid contained 2 per cent of a neutral impurity. These were removed by dissolving the acids in an alkaline solution of 50 per cent ethanol and washing three times with petroleum ether. The alcoholic solution was then acidified and the fatty acid extracted with petroleum ether which was evaporated to dryness. Water was added, the pH adjusted to 9.0 with ammonium hydroxide and the solution heated in a steam bath until clear (13). The final concentration for both acids was 5×10^6 cpm per ml. Sodium octanoate (6.75×10^5 cpm per μMole) contained no radioactive neutral impurity, and was brought into solution in a similar manner.

Tween "80"® (polyoxyethylene sorbitan mono-oleate) was prepared as a 10 per cent (v/v) aqueous solution. Free fatty acids were removed by the method of Minard (18). Coenzyme A was obtained from Pabst Laboratories, Milwaukee, Wisconsin, or Sigma Chemical Company, St. Louis, Missouri, and solutions were freshly prepared before each experiment. L-α-glycerophosphate was a gift of Dr. Erich Baer.

Female albino rats (Charles River Laboratories) weighing 150 to 250 Gm. were fed on Purina® Chow. They were killed by a blow on the head. The small intestine was washed through with ice cold isotonic saline and the mucosa obtained by scraping the serosa with a spatula. A 5 per cent homogenate was prepared with a tissue grinder (Potter-Elvehjem, Teflon Pestle) in 0.15 M KCl and filtered through a double layer of absorbent gauze. Cell debris and nuclei were removed by spinning at $600 \times G$ for 15 minutes. When more concentrated homogenates were used, this step was difficult to perform due to the presence of excessive insoluble mucus. The protein concentration of the supernatant fraction was measured spectrophotometrically (19) and then the fraction was diluted with 0.15 M KCl to a final concentration of less than 1 mg. per ml.

Human intestinal mucosa was obtained from 25 patients of whom 9 were women and 16 men.[1] Their ages ranged from 21 to 68 years. Histologically normal sam-

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‡ Lederle Traveling Fellow of the British Medical Research Council. Present address: Department of Medicine, Royal Free Hospital, London, England.

[1] We are indebted to Dr. John A. Benson, Jr. for his help and assistance in obtaining the human intestinal tissue for these experiments.

ples were obtained from the jejunum of six patients of whom five were undergoing surgery—four for peptic ulcer and one for chronic pancreatitis. In the sixth patient, who had postgastrectomy steatorrhea, a biopsy of the afferent jejunal loop was taken with a Shiner tube (20). Histologically normal terminal ileum was obtained from one patient at operation for carcinoma of the cecum and through a biopsy tube from six patients who had an ileostomy for chronic ulcerative colitis. Histologically normal colon or rectum was obtained from four patients at operation for large bowel tumors and from two patients by using biopsy forceps at routine sigmoidoscopy. Jejunal tissue was removed from six patients with idiopathic steatorrhea—one at operation and five by using a Shiner biopsy tube. This tissue showed the usually accepted histological changes found in this disease (20, 21). Immediately after it was obtained the specimen was placed in 2 to 3 ml. of ice cold 0.15 M KCl and, as soon as possible, homogenized in a tissue grinder (Potter-Elvejhem type). It was then centrifuged at 600 × G in the cold and the supernatant solution assayed for its enzymatic activity.

Incubations of the 600 × G supernatant fractions from the rat and human homogenates were carried out in Pyrex® centrifuge tubes for 30 minutes at 37° C. in air without agitation. The standard incubation mixture contained 0.5 ml. Tris-maleate [2] buffer, 0.5 M, pH 7.0; $MgCl_2$, 10 μMoles; potassium fluoride, 25 μMoles; 0.1 ml. of a 10 per cent solution of Tween "80"®; ATP, 10 μMoles, CoA, 0.5 μMole, C^{14}-labeled fatty acid, 100 mμMoles and 0.5 ml. homogenate containing less than 0.5 mg. protein. The final volume of the mixture was 1.5 ml. A 1 ml. aliquot was removed after incubation and the total lipid extracted by adding it to 20 ml. chloroform-methanol (2:1) (22). After standing 30 minutes, the mixture was filtered through Whatman No. 1 filter paper. The filtrate was evaporated and the lipids taken up in 10 ml. of petroleum ether (B.P. 30 to 60° C.) The free fatty acids and monoglycerides were removed from the petroleum ether by extracting three times with 25 ml. of 50 per cent ethanolic 0.25 N KOH (23). A 2 ml. aliquot of the petroleum ether was then added to 10 ml. of toluene containing 0.01 per cent *p-bis*[2-(5-phenyloxazolyl)]-benzene and 0.3 per cent 2,5-diphenyloxazole (Pilot Chemicals Inc., Waltham, Mass.) and the radioactivity measured in a Packard Liquid Scintillation Spectrometer. In each experiment the control consisted of a tube to which the radioactive fatty acid was added immediately prior to extraction, and on the average less than 0.3 per cent of the radioactivity remained in the petroleum ether. This blank value was subtracted before expressing the results as micromoles of C^{14}-labeled fatty acid incorporated into neutral fat. Duplicate determinations usually varied less than 10 per cent.

In later experiments, when the distribution of the radioactive fatty acid among the various lipid fractions was measured, an alternative extraction procedure was used. This was used because it was observed that washing with alkaline ethanol not only removed monoglycerides (23) but also changed the behavior of the lipid on silicic acid columns. This alternative extraction procedure consisted of supersaturating the incubation mixture with sodium sulfate crystals followed by the addition of about 10 mg. of sodium lauryl sulfate. The mixture was made alkaline with 0.1 ml. 5 N KOH and extracted four times with 3 ml. of ethyl ether (24). This wet ether extract was passed through a 5 Gm. Amberlite IRA-400 column (23) to remove any remaining free fatty acids. The column was washed with a further 30 ml. of wet ether and the combined ether washings were evaporated to dryness. The lipid was taken up in petroleum ether, washed with 0.1 M KCl and an aliquot counted in the liquid scintillation spectrometer.

The products were analyzed on a small silicic acid column using a modification of the method of Barron and Hanahan (25). Two Gm. of silicic acid (Bio-Rad Laboratories, Berkeley, Calif.) was baked overnight at 120° C. After cooling, 1 Gm. of Hyflo® supercel (Johns Manville Co.) was added and the mixture washed in a frittered glass funnel with 50 ml. each of anhydrous ethyl ether, 15 per cent benzene in hexane, and finally hexane. The hexane was prepared according to Barron and Hanahan (25). The slurry was suspended in 30 ml. hexane and poured into an 8 mm. diameter column. After initial settling, the column was packed by gentle water suction. A small disc of ether-extracted filter paper was placed on top of the silicic acid and the column washed with a further 20 ml. of hexane. The lipid to be analyzed was applied in 1 ml. of hexane. The developing solvents were 1 per cent benzene in hexane, 3 per cent ethyl ether in hexane, 30 per cent ethyl ether in hexane and ethyl ether. The column was finally stripped with 50 ml. methanol. The column was initially standardized with mono-, di-, and triolein and the eluates checked by chromatography on silicone impregnated paper (26).

Triglyceride labeled with palmitate-1-C^{14} was prepared by pooling the labeled products of numerous incubations and isolating the triglyceride on a silicic acid column. This was emulsified by the method of Borgström (27). Sodium taurocholate was obtained from Pfanstiehl Laboratories, Waukeegan, Illinois and cholic acid from Matheson, Coleman, and Bell, Norwood, Ohio. These showed one bile acid spot on paper chromatograms (28).

RESULTS

Properties of the system and cofactor requirements

Under the conditions described, homogenates of the mucosa of rat small intestine were very active in their ability to incorporate palmitate-1-C^{14} into neutral fat. The palmitate incorporation was proportional to homogenate protein up to a protein content of 0.5 mg. Above this amount the curve usually leveled off (Figure 1) and in experiments where the protein exceeded 5 mg. (not shown in

[2] Tris = Tris(hydroxymethyl)aminomethane.

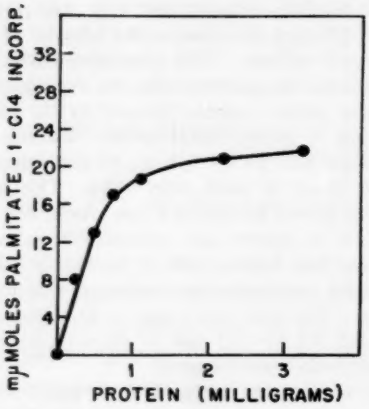

FIG. 1. THE EFFECT OF INCREASING AMOUNTS OF A HOMOGENATE OF RAT SMALL INTESTINAL MUCOSA ON THE INCORPORATION OF PALMITATE-1-C^{14} INTO NEUTRAL FAT

The system contained 0.5 ml. Tris-maleate buffer 0.5 M, pH 7.0, 10 μMoles $MgCl_2$, 25 μMoles KF, 0.1 ml. Tween "80"® (10 per cent solution), 0.5 μMoles Coenzyme A (CoA), 10 μMoles adenosine triphosphate (ATP), palmitate-1-C^{14}, 5×10^5 cpm (0.1 μMole) and varying amounts of homogenate protein as indicated in a final volume of 1.5 ml. Incubation was at 37° C. for 30 minutes.

Figure 1) reduction in the incorporation of label actually occurred. Most of the activity in the homogenate was lost by storing at either − 10° C. or + 4° C. for 24 hours.

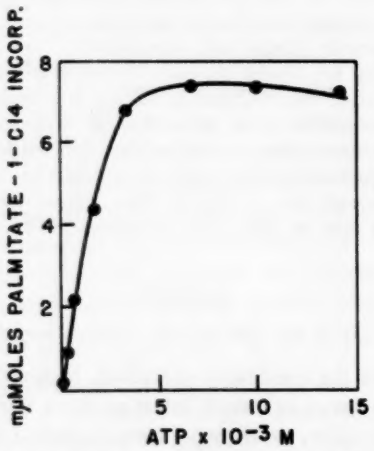

FIG. 2. THE EFFECT OF ATP CONCENTRATION ON THE INCORPORATION OF PALMITATE-1-C^{14} WITH A HOMOGENATE OF RAT SMALL INTESTINE

Standard incubation procedure was used except for the variation of ATP. Each tube contained 0.18 mg. homogenate protein.

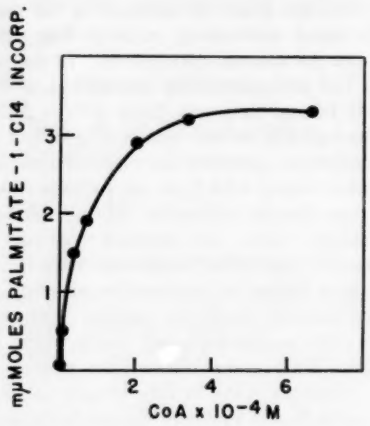

FIG. 3. THE EFFECT OF CoA CONCENTRATION ON THE INCORPORATION OF PALMITATE-1-C^{14} (RAT)

Standard incubation procedure was used except for the varying amounts of CoA. Each tube contained 0.19 mg. homogenate protein.

ATP was essential for the incorporation of palmitate-1-C^{14} into neutral fat and only negligible activity was observed in its absence. The optimal concentration of ATP in this system was 6.6×10^{-3} M (Figure 2). Coenzyme A was also necessary and its optimal concentration in the standard incubation mixture was 3.3×10^{-4} M (Figure 3). As might be expected, magnesium was required and it is seen in Figure 4 that optimal incorpora-

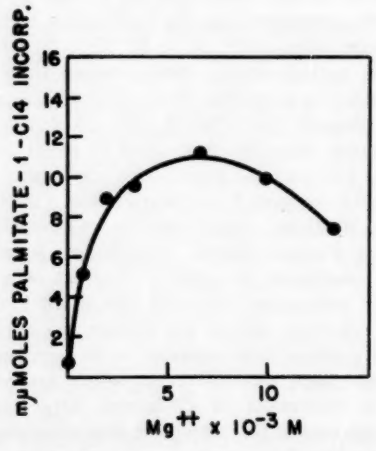

FIG. 4. THE EFFECT OF MAGNESIUM CHLORIDE CONCENTRATION ON THE INCORPORATION OF PALMITATE-1-C^{14} (RAT)

Standard incubation procedure was used except for the variation of Mg^{++} concentration as indicated. Each tube contained 0.36 mg. homogenate protein.

tion of label occurred at the magnesium ion concentration of 6.6×10^{-3} M. Higher concentrations inhibited the reaction.

There was a linear relationship between the time of incubation and palmitate incorporation until the reaction was complete. This took up to 50 minutes for homogenates of high activity, but the reaction was complete within 20 minutes with homogenates of lower activity. The pH optimum for the reaction was found to be a broad one, between 7 and 8 (Figure 5).

Tween "80"® was initially added to the mixture to maintain the lipid components in solution and, indeed, the presence of such an agent has often been necessary to demonstrate some aspects of lipid metabolism *in vitro* (29). In the present

TABLE I

*The effect of potassium fluoride on palmitate-1-C¹⁴ incorporation into neutral fat by homogenates of rat small intestinal mucosa**

Additions			
KF (1.6×10^{-3} M)	ATP	Tween "80"® (final concentration)	Palmitate-1-C¹⁴ incorporated
	μMoles	*% ×10²*	*mμMoles*
0	1	66.0	1.4
+	1	66.0	1.2
0	10	66.0	3.6
+	10	66.0	3.4
0	10	0.66	0.9
+	10	0.66	2.4

* Standard incubation conditions except for variation of potassium fluoride, adenosine triphosphate (ATP) and Tween "80"®. There was 0.40 mg. of homogenate protein in each tube.

system the stimulation of palmitate incorporation into neutral fat by Tween "80"® was impressive (Figure 6), but this stimulation could be partially explained by the inhibitory effect of Tween "80"® on a lipase present in the homogenate. Evidence for this is afforded indirectly by experiments with potassium fluoride and more directly by experiments employing C¹⁴-labeled triglyceride.

Stimulation of palmitate incorporation by fluoride would most likely be due either to the inhibition of an ATPase or of a lipase (30). The results shown in Table I favor the lipase hypothesis. If fluoride stimulation were due to inhibition of ATPase one would expect a stimulation in the presence of suboptimal amounts of ATP and optimal amounts of Tween "80"®. This was not observed. In contrast, when the system contained suboptimal amounts of Tween "80"® and optimal ATP, the stimulation by fluoride was nearly threefold. This increased incorporation in the presence of fluoride could be explained by lipase inhibition,

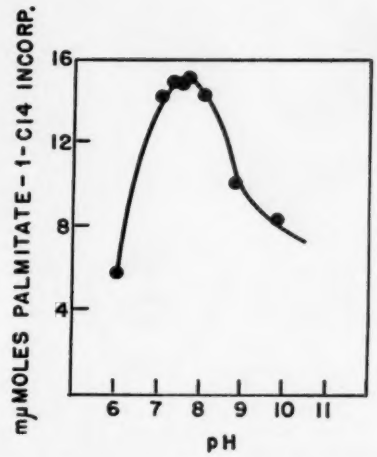

FIG. 5. THE EFFECT OF pH ON THE INCORPORATION OF PALMITATE-1-C¹⁴ (RAT)

Standard incubation procedure was used except that the incubation time was 100 minutes. Between pH 6 and 8, Tris-maleate buffer was used, while glycine buffer was used for pH 9 and 10. Each tube contained 0.15 mg. homogenate protein. The pH of the system was measured after incubation with a glass electrode at room temperature.

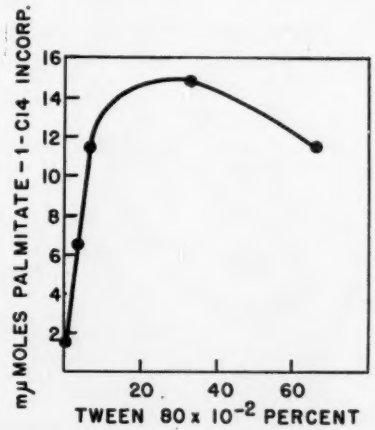

FIG. 6. THE EFFECT OF TWEEN "80"® ON THE INCORPORATION OF PALMITATE-1-C¹⁴ (RAT)

The standard incubation procedure was used except for the variation in the concentration of Tween "80"®. Each tube contained 0.25 mg. homogenate protein.

TABLE II

*Lipase activity in a homogenate of rat small intestinal mucosa**

Additions	Neutral fat	Glyceride fraction	
		Tri	Mono +di
	cpm	%	%
1. None (4° incubation)	650	95	5
2. None (37° incubation)	60†		
3. Tween "80"® + KF	530	68	32
4. Tween "80"® + KF + taurocholate	270	33	67

* Each flask contained 0.5 ml. Tris-maleate buffer, 0.5 M, pH 7.0; 0.5 ml. emulsion of palmitate-1-C^{14}-labeled triglyceride; $MgCl_2$, 5×10^{-3} M; and 2.2 mg. homogenate protein. Tween "80"®, 0.5 per cent; KF, 1.25×10^{-2} M; and taurocholate, 2×10^{-3} M were added as indicated. Final volume was 2.0 ml. and the mixture was incubated for 60 minutes at 37° C. After incubation the lipids were extracted and any liberated free fatty acid removed by passing through an IRA-400 column. The remaining lipid was separated into tri- and lower glycerides on a silicic acid column.

† Too few counts for accurate glyceride analysis.

resulting from the additive effect of the fluoride and Tween "80"® (18). The results of a more direct experiment are shown in Table II. Lipase activity was demonstrated when palmitate-1-C^{14}-labeled triglyceride was incubated with a homogenate of small intestinal mucosa at 37° C. This was not observed in the control tube incubated at 4° C. In the presence of Tween "80"® (0.5 per cent) and potassium fluoride (1.25×10^{-2} M) the breakdown of the labeled triglyceride was largely but not completely prevented for there was a slight loss of total counts and an appearance of labeled lowered glycerides. This protection was partially overcome by the addition of taurocholate, a known lipase activator (*27*).

The addition to the standard incubation mixture of either L-α-glycerophosphate (1 μMole), glucose (3 μMoles), glycerol monostearate (1 μMole) or glycerol diolein (1 μMole) produced no consistent increase in the incorporation of palmitate-1-C^{14} into neutral fat.

Results using homogenates of human intestinal tissue were comparable, both qualitatively and quantitatively, to those obtained with the rat intestine. Typical findings showing the dependence upon ATP, CoA and magnesium, as well as the effect of Tween "80"® and potassium fluoride, are shown in Table III. Similar to the observations with the rat homogenates, there was a linear relationship between the esterification of palmitate-1-C^{14} and the amount of homogenate protein in the range studied, which was up to 0.5 mg. (Figure 7). It will be seen that with homogenates of mucosa from patients with idiopathic steatorrhea, the incorporation was significantly lower but a linear relationship between homogenate protein and palmitate esterification still obtained.

TABLE III

*The effect of cofactors in the esterification of palmitate-1-C^{14} by homogenates of human small intestinal mucosa**

Incubation mixture	Palmitate-1-C^{14} esterified
	mμMoles
Complete	5.1
Minus ATP	0.5
Minus CoA	0.4
Minus Mg^{++}	0.7
Minus Tween "80"®	2.9
Minus KF	4.2

* The incubation mixture contained 0.5 ml. Tris-maleate buffer, 0.5 M, pH 7.0; ATP, 10 μMoles; CoA, 0.5 μmole, $MgCl_2$, 10 μMoles; 0.1 ml. Tween "80"® (10 per cent solution); KF, 25 μMoles; and palmitate-1-C^{14}, 100 mμMoles (5×10^5 cpm). Homogenate protein was 0.18 mg. and the final volume 1.5 ml. Incubation was for 30 minutes at 37° C.

Analysis of lipid products formed

The labeled esterified lipid formed during an incubation was stable to 0.1 N KOH at 37° C. for

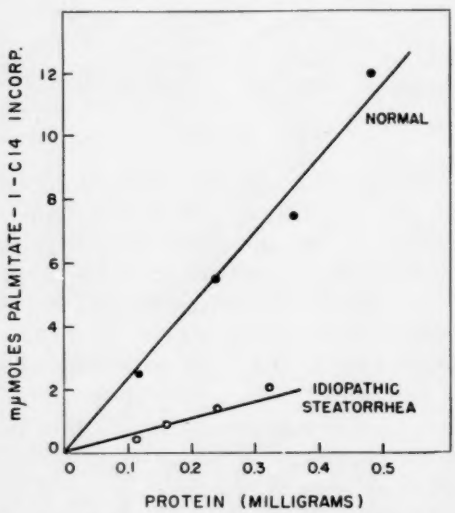

FIG. 7. THE EFFECT OF INCREASING AMOUNTS OF A HOMOGENATE OF HUMAN JEJUNAL MUCOSA (NORMAL AND IDIOPATHIC STEATORRHEA) ON THE INCORPORATION OF PALMITATE-1-C^{14} INTO NEUTRAL FAT

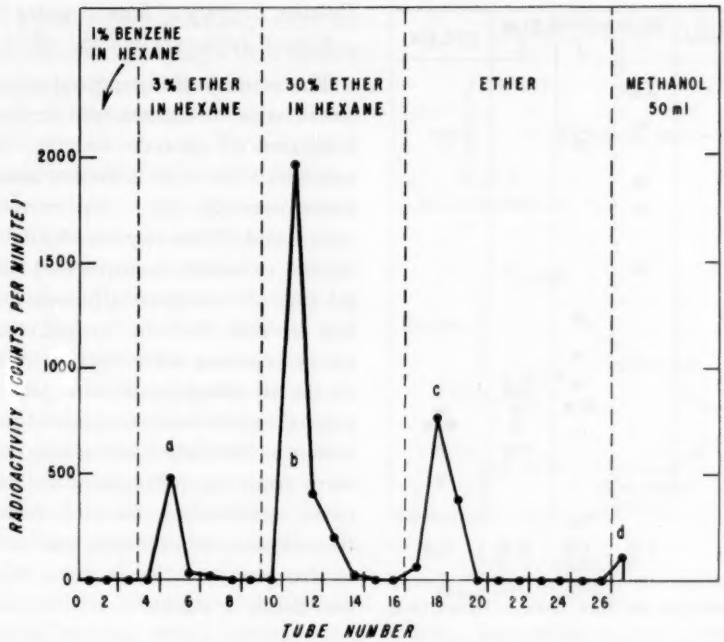

FIG. 8. RESULTS OF SILICIC ACID CHROMATOGRAPHY OF LABELED ESTERIFIED PRODUCTS OBTAINED FROM AN INCUBATION WITH A HOMOGENATE OF RAT SMALL INTESTINE

Each tube contained 6 ml. of eluate. Peak **a** (triglyceride), 12 per cent total radioactivity; Peak **b** (diglyceride), 58 per cent total radioactivity; Peak **c** (monoglyceride), 27 per cent total radioactivity; Peak **d** (phospholipid), 3 per cent total radioactivity.

30 minutes and thus did not merely represent the formation of palmityl-CoA (13). The result of a typical analysis of the products obtained from an incubation with a rat small intestinal homogenate is shown in Figure 8. Quantitatively similar results were obtained with homogenates of human intestinal tissue. It will be observed that most of the label was incorporated into the glyceride fractions. In analyses of lipid isolated from some other incubations, about 1 per cent of the radioactivity was eluted before the glyceride fractions and probably represented cholesterol esters. After the glyceride peaks were eluted, a variable but small proportion of labeled lipid (3 to 8 per cent) remained on the column which could be removed with 50 ml. of methanol. No attempt was made to identify this fraction, but on the basis of its polarity it was assumed to be phospholipid. The glyceride fraction always consisted of a mixture of tri-, di- and monoglyceride, of which the diglyceride usually predominated. Similar results were obtained by chromatography on silicone impregnated paper, and it is of interest that the proportion of different glycerides in the sample, as shown by the degree of staining of the paper with iodine vapor, was roughly proportional to the distribution of the radioactivity. The ratio of glycerides may be altered under certain conditions (Table IV). Thus, when taurocholate (1.3×10^{-3} M) was added to

TABLE IV

*The effect of taurocholate on palmitate-1-C^{14} incorporation into glyceride fractions**

Incubation mixture	Total palmitate-1-C^{14} incorporated	Glyceride fraction		
		Tri	Di	Mono
	mμMoles	%	%	%
Complete	13.2	28	49	23
Plus taurocholate (1.3×10^{-3} M)	5.2	13	22	65

* Standard incubation procedure was used. Each tube contained 0.40 mg. protein.

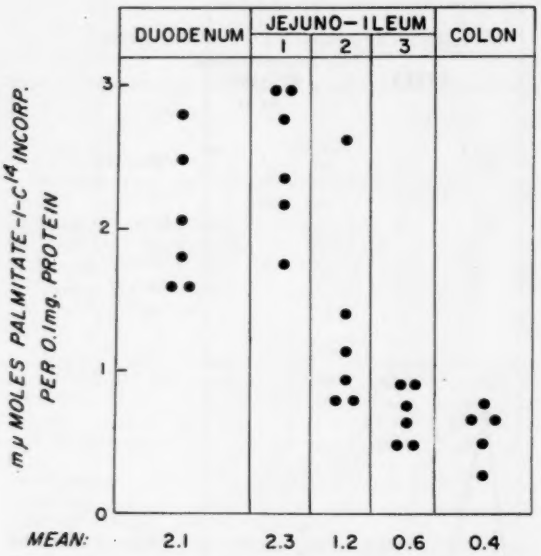

FIG. 9. A COMPARISON OF PALMITATE-1-C^{14} INCORPORATION BY HOMOGENATES OF RAT SMALL INTESTINAL SEGMENTS AND COLON

The intestine was washed with saline and homogenates were prepared from the mucosa of the duodenum, the jejuno-ileum (divided into three equal segments) and the colon. The numbering of the jejuno-ileal segments is from above downward. The standard incubation procedure was used and less than 0.4 mg. homogenate protein was present in the mixture. Each point represents the mean of duplicate determinations.

the incubation, the total incorporation of palmitate-1-C^{14} was depressed and a significantly greater proportion of monoglycerides was isolated. Similar results were obtained with cholate (1.3×10^{-3} M). These effects may be explained in part by the stimulation of lipase activity by these bile salts.

Effect of fatty acid chain length

In view of the difference in the intestinal absorption of fatty acids depending upon their chain lengths (7–11), the ability of the rat and human homogenates to incorporate C^{14}-labeled octanoate, palmitate and stearate into neutral fat was compared. Taking palmitate incorporation arbitrarily as 100, the incorporation of stearate was 44 ± 9 (mean $\pm$ S. E.) and of octanoate 1.2 ± 0.4, using rat intestinal tissue. In homogenates prepared from the jejunum of three human subjects the mean incorporation of stearate was 29 and of octanoate 1.3.

Relative activity of homogenates from different intestinal segments and the effect of abnormal villi

The activity of homogenates prepared from different sites of the rat and human intestine were compared to observe whether the results would correlate with what is known about the sites of fat absorption (31–33). The results shown in Figures 9 and 10 are expressed as the amount of C^{14}-labeled palmitate incorporated into neutral fat by 0.1 mg. of homogenate protein in 30 minutes. It will be seen that, in the rat, the duodenum and upper jejunum were four to five times as active as the terminal ileum or colon. Normal human jejunal tissue was more active than terminal ileum, which in turn was more active than colon. Specimens from the jejunum of six patients with idiopathic steatorrhea, all with histologic criteria of this disease, exhibited a low activity comparable to that usually found in tissue taken from the normal ileum or colon.

DISCUSSION

The system described above for the incorporation of long chain fatty acids into glycerides by homogenates of rat and human intestinal mucosa is similar to that observed in rat liver by Stein, Tietz and Shapiro (15, 16). In both instances incorporation rather than net synthesis was studied. The dependence of this incorporation upon CoA and ATP suggests that the fatty acid is first activated to the acyl-CoA derivative before

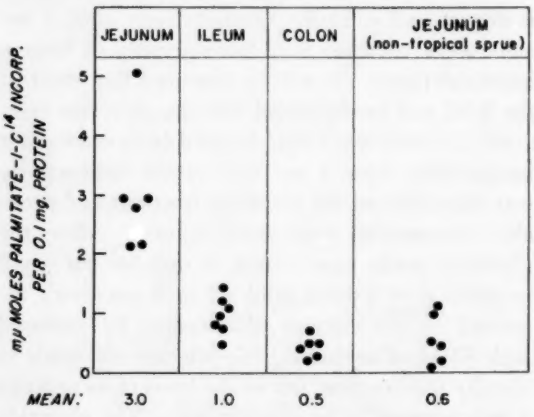

FIG. 10. A COMPARISON OF PALMITATE-1-C^{14} INCORPORATION BY HOMOGENATES OF NORMAL HUMAN JEJUNUM, ILEUM AND COLON AND THE JEJUNUM OF PATIENTS WITH IDIOPATHIC STEATORRHEA

being esterified. These cofactor requirements indicate that the incorporation of fatty acid cannot be explained simply by the synthetic action of a lipase and, indeed, to demonstrate the reaction lipase had largely to be inhibited by Tween "80"® and potassium fluoride. The origin of this lipase is unknown. The studies on rat intestinal tissue (Table II) suggest that it represents an intramucosal rather than a pancreatic enzyme, for the intestine had been repeatedly washed before being homogenized and cell debris, which included cell walls, had been removed from the homogenate by spinning at 600 × G. No experiments were performed on human tissue to demonstrate lipolytic activity. However, the even distribution of label among the glyceride fractions after a standard incubation suggests that a mucosal lipase was probably present in the homogenates of the human biopsy specimens.

The system observed by Favarger and Gerlach in rat small intestinal mucosa, which incorporated palmitate-1-C^{14} into neutral fat, differed from ours (12). It was of lower activity; there was no dependence upon CoA or ATP; and the pH optimum was 5. Thus their results may have represented the synthetic action of a lipase. The differences between the two systems can perhaps be explained by the fact that Favarger and Gerlach did not inhibit lipase activity with Tween "80"® and potassium fluoride, and possibly by their use of far more concentrated homogenates. Such homogenates depressed palmitate incorporation under our experimental conditions.

Kennedy has expanded the early observations of Kornberg and Pricer (13, 14) and demonstrated a series of reactions relating the synthesis of triglycerides and phospholipids (29). These are represented in Figure 11. One will notice that there is no place in this scheme for the formation of monoglycerides and that phosphatidic acid is the precursor of 1,2-diglyceride, which in turn may become either triglyceride or phospholipid. The fact that analyses of the labeled lipid formed in our experiments showed an appreciable quantity of monoglycerides in no way negates the Kennedy scheme, for it will be noted in Table II that the lipolytic activity present in the homogenate was not completely inhibited by the addition of Tween "80"® and potassium fluoride. Thus, the labeled lipid formed during an incubation may be the result of both neutral fat formation on the one hand and unsuppressed lipolysis on the other. This is supported by the experiments in which taurocholate, a known lipase activator, was added to the incubation medium (Table IV). This resulted in a diminished total incorporation of palmitate into neutral fat but in an increased proportion of labeled monoglyceride. A contributing factor to the lowered incorporation of palmitate in the presence of taurocholate may have been the inhibition of acyl-CoA formation by the bile salt (13).

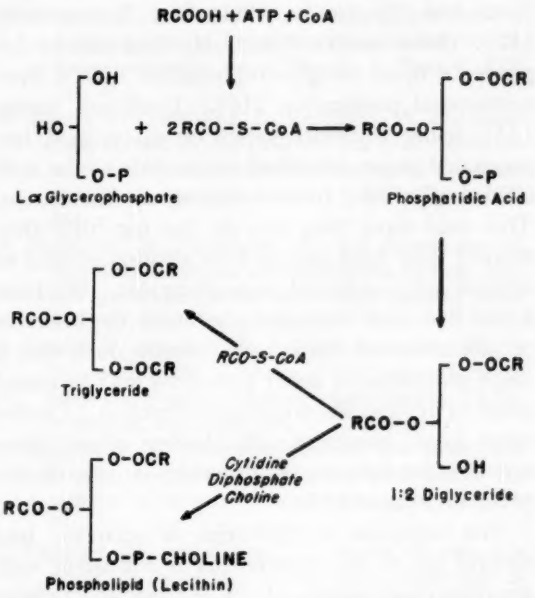

FIG. 11. PATHWAYS FOR THE ENZYMATIC SYNTHESIS OF PHOSPHOLIPID AND TRIGLYCERIDE

This scheme has been outlined by Kennedy (29). R = long chain fatty acid.

Buell and Reiser have recently studied the incorporation of C^{14}-fructose-1,6-diphosphate into glyceride-glycerol using fortified concentrated homogenates of hog intestinal mucosa (34). They concluded that L-α-glycerophosphate was the immediate glyceride-glycerol precursor which is in accordance with Kennedy's scheme. They also indicated that the addition of L-α-glycerophosphate to the homogenate was necessary before incorporation of palmitate-1-C^{14} into triglyceride could be observed. In our rat intestinal homogenate system L-α-glycerophosphate did not have to be added nor did its addition cause any stimulation. Results similar to ours have been reported by Stein,

Tietz and Shapiro in whole liver homogenates (15). These workers could only demonstrate dependence upon L-α-glycerophosphate with a liver microsomal preparation (16). Buell and Reiser (34), using chromatography on silicic acid impregnated paper, identified triglyceride as the only labeled glyceride formed during an incubation. This may have been due to the fact that they washed their lipid sample with alkaline ethanol to remove fatty acids and monoglycerides. We have found that such treatment also alters the behavior of glycerides on silicic acid columns such that a large proportion of lower glycerides may be eluted prior to or with the triglyceride fraction. On the other hand, treatment with alkaline ethanol does not alter the behavior of glycerides on silicone impregnated paper (35).

The negligible incorporation of octanoate into neutral fat in our experiments is consistent with previous observations (3, 10, 11) that short chain, in contrast to long chain fatty acids, pass unesterified into the portal vein during absorption. This difference in the behavior of short chain fatty acids, both *in vivo* and *in vitro,* may be related either to their greater water solubility or to enzyme specificity. Greater water solubility might reduce their contact with the enzymes involved in activation or esterification. On the other hand, it is possible that one or both of these enzyme systems in the mucosa are specific and act only on long chain fatty acids. Such enzyme specificity has been observed by Kornberg and Pricer (13, 14) in guinea pig liver microsomal preparations. They found that the activation of octanoate was approximately half that of palmitate, but that the esterification of octanoate was only minimal. The relative status of these enzyme systems in the intestine is as yet unknown. In our rat and human experiments, stearate incorporation into neutral lipid was also less than that of palmitate. This correlates with but is probably not causally related to the fact that in the rat stearate is normally absorbed less efficiently than palmitate (36). Again the relative importance of fatty acid solubility and enzyme specificity in explaining the *in vivo* and *in vitro* behavior of stearate remains to be established.

Further evidence that the system we have observed may be involved in fatty acid absorption is provided by the experiments in which the activity of the mucosa from different segments of the rat and human intestine was compared (Figures 9 and 10). The fact that homogenates from the upper small intestine were more active in incorporating palmitate than those from the lower intestine or colon is compatible with the observations that fat absorption in the rat usually occurs in the duodenum and jejunum (31, 32) and the findings of Borgström, Dahlqvist, Lundh and Sjovall (33) that the major area of fat absorption in man is in the upper small intestine. Since the enzymatic activity of the mucosa is probably in the villous epithelial cells, a homogenate containing less villous epithelium per milligram of protein would be expected to be less active. The difference in enzymatic activity along the intestine may thus be due to an *anatomic* factor and may be explained by a decrease in the number of villi as one proceeds from the upper to the lower intestine (37). On the other hand, it is possible that the gradation of activity may in part represent a *functional* differentiation of the cells along the intestine. An example of such specialization of intestinal cell function is the observation that vitamin B_{12} absorption occurs primarily in the ileum (38). Our data do not allow us to conclude at this time whether the differences in palmitate incorporation along the intestine are on an anatomic or a functional basis, or both. It is of interest that the jejunum of patients with idiopathic steatorrhea possessed a very low activity, comparable to that of the normal ileum or colon. Since such jejunum has flattened villi with abnormal epithelial cells, these results may again be a reflection either of a diminution in the proportion of villous epithelial cells or of altered mucosal cell function. It cannot be concluded, nor do we propose that the low activity observed in patients with idiopathic steatorrhea is the primary basis for their malabsorption of fat.

The data presented indicate that the mucosal epithelium of the rat and human intestine is most active in the incorporation of long chain fatty acids into neutral fat. The correlation between the properties of the system described by us and previous physiologic observations (3, 33) lends support to the view that a mechanism similar to that demonstrated *in vitro* is involved in the esterification of long chain fatty acids during their absorption by the intestine.

SUMMARY

1. A very active system has been demonstrated in cell-free homogenates of rat and human intestinal mucosa which incorporates palmitate-1-C^{14} into neutral fat. This incorporation is dependent upon Coenzyme A, adenosine triphosphate and magnesium ions. Short chain fatty acids are not incorporated by this system.

2. Lipase activity had to be suppressed with Tween "80"® and potassium fluoride before significant incorporation could be demonstrated.

3. The labeled neutral fat formed *in vitro* was a mixture of mono-, di- and triglyceride and probably represented a balance between the incorporation of palmitate-1-C^{14} into neutral fat and lipolysis.

4. In both rat and human experiments, homogenates prepared from the duodenum or jejunum, which are the usual sites of fat absorption, were four to five times as active as those from the ileum or colon. Mucosa from the jejunum of patients with idiopathic steatorrhea was found to have a greatly diminished capacity to esterify palmitate-1-C^{14}.

5. It is suggested that a mechanism similar to the one described *in vitro* is involved in the esterification and absorption of long chain fatty acids by the intestine *in vivo*.

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DETERMINANTS OF THE FLOW AND COMPOSITION OF BILE IN THE UNANESTHETIZED DOG DURING CONSTANT INFUSIONS OF SODIUM TAUROCHOLATE *

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Physiological factors affecting the flow and composition of bile may be divided into two general categories. First, the availability of bile salts (by hepatic synthesis and by reabsorption from the bowel) has a significant influence on the rate of bile production. The choleretic effect of natural bile salts and the phenomenon of enterohepatic circulation of these compounds were first demonstrated by Schiff in 1870 (1). These observations have been confirmed by many workers since that time. It has been estimated that 85 to 90 per cent of the bile salt excreted in the bile is reabsorbed and returned to the liver (2, 3). For this reason the entry of bile itself into the duodenum provides a major stimulus for further bile production. Second, factors independent of the availability of bile salts can affect bile production. This category would include, for example, neural stimuli (4, 5), humoral agents such as secretin (6–8) and changes in vascular perfusion (5).

During any investigation of biliary secretion in which bile is removed and not replaced, achievement of a steady state is thwarted by progressive depletion of bile salts which would normally undergo enterohepatic circulation. In the present studies this problem has been circumvented by constant intravenous infusion of sodium taurocholate. The results substantiate the important role of active bile salt secretion in the process of bile production and also suggest that the flow and composition of bile are modified by the addition of variable amounts of a solution which is similar in certain respects to pancreatic juice.

METHODS

All studies were conducted on four trained adult female mongrel dogs (21 to 30 Kg.) which had been prepared several months previously by splenectomy, cholecystectomy and installation of a Thomas duodenal fistula apparatus (9). Food and water were withheld for 15 hours prior to each study. The fistula was opened and a No. 5 or No. 6 Fr. olive-tipped ureteral catheter inserted into the ampulla of Vater and advanced about 5 cm. into the common bile duct. The animal was then placed upright in a sling. Bile was withdrawn by gentle aspiration using an oiled tuberculin syringe, and collected under mineral oil in graduated tubes. By noting the volume collected, frequent estimations of bile flow were obtained (at intervals of 2 to 10 minutes depending on the flow). Approximately 4 ml. of bile was required to carry out all of the analytical procedures, and specimens were selected for analysis which represented periods of relatively constant flow in order to minimize "dead space" errors (due to the volume of bile in the hepatic ducts and the catheter). Specimens were rejected when obvious changes in flow occurred during their collection or during the collection of the preceding 2 ml. of bile. The figure of 2 ml. represents a rough estimate of "dead space" based on our observation of the approximate volume of bile one must collect before noting the presence of high concentrations of intravenously injected sulfobromophthalein sodium.

The "sodium taurocholate" used for intravenous infusion was a commercial preparation [1] in which it was found that bile salt accounts for approximately 75 per cent of the total solids. Approximately 25 per cent of the bile salt is derived from dihydroxy rather than trihydroxy cholanic acid, but virtually all of it appears to be conjugated with taurine since only a trace of glycine was detected after hydrolysis. This material was dissolved in normal saline and passed through a Seitz filter. In a majority of studies a 1 per cent solution was infused through a nylon intravenous catheter at approximately 1 ml. per minute using a Bowman pump. In a few of the later studies a 2 per cent solution was infused at 1.5 ml. per minute. The rates of bile salt infusion stated in the text were calculated by multiplying the calibrated rate of delivery of the Bowman pump by the measured concentration of bile salt in the infusion mixtures. Hemolysis was minimal or, in most instances, undetectable after infusions lasting up to five hours.

Secretin [2] was diluted to 10 units per ml. in normal

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[1] Purchased from Nutritional Biochemicals Corp.

[2] Supplied by Eli Lilly & Co. through the courtesy of Dr. James B. Hammond.

saline and administered intravenously in doses of 100 units. Acetazolamide [3] was diluted to 100 mg. per ml. in normal saline and administered intravenously in doses of 65 mg. per Kg. Intraduodenal infusions of 0.05 N hydrochloric acid in normal saline were administered at 7 ml. per minute by way of a catheter inserted through the duodenal fistula and advanced about 5 cm. distal to the fistula.

The pH was measured anaerobically in each bile specimen immediately after collection by means of a Cambridge pH apparatus. Total CO_2 content was determined by the method of Van Slyke and Neill (10) and bicarbonate concentration calculated by the Henderson-Hasselbalch equation assuming a pK_a of 6.1. Sodium and potassium were measured by flame photometry, chloride by the method of Cotlove, Trantham and Bowman (11), and freezing point depression in a Fiske osmometer. Bile acid concentrations were determined by the method of Mosbach, Kalinsky, Halpern and Kendall (12).[4] This method has yielded a recovery of 96.5 ± S.D. 3.3 per cent in our laboratory. Although appreciable amounts of dihydroxy bile acids were found in the bile, the total concentration of conjugated bile acid will be referred to as "taurocholate" hereafter for convenience. Estimations of amylase were carried out on bile and on serial dilutions of duodenal juice in water and in bile by mixing 0.2 ml. of the test material with 0.4 ml. of a 0.5 per cent starch solution in pH 7.0 phosphate buffer and incubating in a 37° C. water bath for two minutes. A positive test consisted of absence of blue color on addition of iodine. When applied either to pure pancreatic juice or to pancreatic juice diluted in bile, this method has proven to be equally sensitive for the detection of pancreatic amylase.

RESULTS

A. Relationship of taurocholate to other electrolytes

Bile acid concentrations were measured on a total of 27 bile specimens of widely varying composition selected at random during the course of these studies. It was found that, within the limits of methodological error, the measured taurocholate concentration $[T^-]$ was equal to the difference between the cations $(Na^+ + K^+)$ and the anions $(Cl^- + HCO_3^-)$. The ratio: $[T^-]/[(Na^+ + K^+) - (Cl^- + HCO_3^-)]$ was equal to 0.98 ± S.D. 0.09 over a range of taurocholate concentrations from 40 to 280 mEq. per L. Thus it appeared that the taurocholate concentration in hepatic bile could be estimated by the formula $[T^-] = [(Na^+ + K^+) - (Cl^- + HCO_3^-)]$ with a degree of accuracy approximately equal to that obtainable by direct measurement of the bile acids. The taurocholate concentrations stated hereafter have all been calculated by this equation.

B. Osmolality

The osmolality of 67 bile samples, as estimated by freezing point depression, showed little variation (299 ± S.D. 11 mOsm. per Kg.) despite marked differences in electrolyte composition, bile flow and time of collection. Thus in the present experimental situation the bile appeared always to be virtually isosmotic with respect to plasma (mean plasma osmolality 303 ± S.D. 4 mOsm. per Kg.). This observation is consistent with the demonstration by Gilman and Cowgill (13) that bile is in osmotic equilibrium with plasma even when the osmolality of the latter is made to vary over a wide range in the dog. Other workers have also found bile to be essentially isosmotic in dogs and in many other species (14).

C. Composition of "common duct bile"

At the time of catheterization approximately 6 to 10 ml. of bile could always be withdrawn from the common bile duct. This bile, referred to hereafter as "common duct bile," must have been secreted over a period of one to several hours prior to the study and retained in the duct system by the normal action of the sphincter of Oddi. The composition of "common duct bile," based on analysis of 16 specimens, was different from that of most subsequent samples of flowing hepatic bile but quite similar to the composition of canine gall bladder bile reported by others (15) and confirmed in two instances in this laboratory. As shown in the example in Figure 1, the concentration of taurocholate in "common duct bile" was typically very high. Taurocholate concentrations in three of the four dogs ranged between 200 and 280 mEq. per L. (average 247 mEq. per L. in 13 specimens), and the concentrations of chloride and bicarbonate were low (range 8.4 to 26.6 and 1.5 to 7.2 mEq. per L., respectively) as was the pH (5.95 to 6.83). However, in one dog, in a total of three specimens, the "common duct" taurocholate concentration never exceeded 134 mEq. per L., and higher concentrations of chloride

[3] Supplied as the sodium salt of Diamox® by Lederle Laboratories through the courtesy of Dr. George K. Morlan.

[4] Cholic acid and deoxycholic acid suitable for analytical standards were supplied by Ames Co., Inc. through the generosity of Dr. Norman L. Heminway.

and bicarbonate were observed (up to 77.5 and 17.7 mEq. per L., respectively). Sodium was always the major cation although "common duct" potassium concentrations reached values as high as 10.6 mEq. per L.

D. Discrepancy between electrolyte concentration and osmolality

In all bile samples there was an obvious discrepancy between the total electrolyte concentration (sum of anions plus cations) and the osmolality. This was most marked in specimens with high taurocholate concentration including those described in the preceding section (see Figure 1). The sum of the measured electrolyte concentrations reached values as high as 579 mMoles per L., and would have been even higher if one had been able to convert from molar to molal concentrations and if other solutes had been included. This discrepancy has been noted by others (13, 15) and is almost certainly due to the tendency of the taurocholate ion to form large aggregates or micelles. According to Pethica and Schulman (16) micelle formation for pure sodium taurocholate begins at concentrations as low as 7.4×10^{-3} M. This tendency is probably enhanced by the presence of lipids in the bile. The structure of these micelles is poorly understood but they are known to include an uncertain quantity of cation (17). Nevertheless the net effect of the aggregration phenomenon in bile is approximately the same as that which one would predict if all of the taurocholate ion were osmotically inactive and all of the other ions had an osmotic activity of 1.0 (15). Thus, over a wide range of biliary taurocholate concentrations the sum of the other major ions was consistent with the observed osmolality: $(Na^+ + K^+ + Cl^- + HCO_3^-) = 295 \pm$ S.D. 16 mEq. per L. There was an apparently linear correlation between taurocholate concentration $[T^-]$ and total cation. In 79 specimens of hepatic and "common duct bile," the coefficient of correlation was + 0.97 and the following regression equation was obtained: $[T^-] = 2.03\ [Na^+ + K^+] - 302$.

E. Bile flow in the absence of bile salt replacement

In initial studies it was found that continuous bile collection over a period of two to five hours resulted in progressive and often profound depression in bile flow and taurocholate concentration. This experience was identical with that reported by Brooks, Buchholz and Gould (18) in the same type of preparation. Because of the resulting difficulty in obtaining adequate bile specimens and interpreting their composition, the technique of continuous intravenous taurocholate infusion was adopted.

F. Control bile flow and composition during constant taurocholate infusion

Sodium taurocholate was infused at a rate of 14.5 μMoles per minute throughout the course of at least five studies on each of the four dogs. Between 16 and 18 bile specimens were obtained in each dog, all at least one hour after onset of infusion. In all collection periods the taurocholate excretion rate was comparable to (or slightly greater than) the rate of administration. Mean excretion rates for the four dogs were 15.7, 15.7, 16.3 and 16.8 μMoles per minute and for the whole group 16.0 $\pm$ S.D. 2.4 μMoles per minute. Thus the intravenous infusion of taurocholate proved to be a satisfactory way of establishing a state of constant and reproducible bile salt excretion rate during which other changes could be more readily interpreted.

There was no progressive diminution in bile flow. However, marked spontaneous variations in bile flow occurred frequently without obvious external stimuli. The range of bile flows encountered in each of the four dogs is shown in Table I (total range 0.074 to 0.347 ml. per minute).

Characteristic alterations in bile electrolyte composition accompanied these spontaneous changes in bile flow. There was a reciprocal relationship between flow and taurocholate concentration, indicating constancy of the excretion rate of this ion. As bile flow increased there was an increase in chloride concentration and a much more striking increase in bicarbonate concentration and pH. This relationship is illustrated in Figure 2 for one of the dogs. In each animal the composition observed at a given rate of bile flow was consistent from one study to another. The concentrations of bicarbonate, chloride, and taurocholate and the pH in the specimens with the highest and lowest spontaneous flows are shown in Table I. In all

TABLE I

Relationship between bile flow and electrolyte composition during intravenous sodium taurocholate infusion at 14.5 μMoles per minute

Dog	Condition	Bile flow	Na^+	K^+	HCO_3^-	Cl^-	Tauro-cholate	pH
		ml./min.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	
	Spontaneous flow	0.074 (min.)	240.4	7.6	16.0	40.5	191.5	7.03
		0.191 (max.)	200.8	6.0	45.7	55.4	105.7	7.61
Norma	Secretin I.V.*	0.315	181.4	5.6	58.3	59.7	69.1	7.76
20.9 Kg.	Intraduodenal HCl	0.230	182.0	5.6	58.0	67.7	62.0	7.75
	Acetazolamide I.V.	0.174	180.0	5.7	25.7	83.3	76.8	7.11
	Spontaneous flow	0.110 (min.)	222.8	8.0	5.7	53.1	161.9	6.71
		0.164 (max.)	179.0	6.9	33.0	61.5	91.4	7.43
Cora	Secretin I.V.*	0.311	177.0	5.6	59.8	75.4	47.3	7.78
24.0 Kg.	Intraduodenal HCl	0.285	176.0	5.7	59.2	71.3	51.1	7.76
	Acetazolamide I.V.	0.355	162.8	4.9	16.9	98.0	52.8	7.04
	Spontaneous flow	0.082 (min.)	242.4	7.2	10.1	38.3	201.0	6.90
		0.347 (max.)	182.4	7.0	50.9	76.0	62.5	7.81
Dali	Secretin I.V.*	0.205	182.4	5.2	48.0	77.0	62.7	7.74
22.7 Kg.	Intraduodenal HCl	0.300	176.0	5.5	52.3	69.9	59.5	7.75
	Acetazolamide I.V.	0.366	173.6	5.4	37.3	88.8	52.9	7.67
	Spontaneous flow	0.140 (min.)	184.8	6.3	8.2	82.8	100.2	6.92
		0.194 (max.)	176.0	6.0	27.5	85.3	69.2	7.44
Isolde	Secretin I.V.*	0.415	181.2	5.9	46.1	102.3	38.7	7.74
30.2 Kg.	Intraduodenal HCl	0.369	181.0	5.6	42.3	95.3	49.0	7.65
	Acetazolamide I.V.	0.340	158.0	5.5	22.0	94.9	46.9	7.29

* The peak bile flows observed after secretin administration in the four dogs were 0.533, 0.440, 0.567 and 0.475 ml. per minute, respectively. It was necessary to discard from 2 to 4 ml. of bile in order to avoid "dead space" errors. The values in the table represent the average flows observed during the collection of the actual specimens on which the analyses were performed.

dogs the composition of bile obtained at intermediate flows followed a pattern similar to that illustrated in Figure 2. The total range of bicarbonate concentrations encountered was 5.7 to 50.9 mEq. per L. and the pH range was 6.71 to 7.81, the higher figures corresponding to a state of marked spontaneous choleresis. Sodium was always the major cation and the concentration of potassium never exceeded 8.0 mEq. per L.

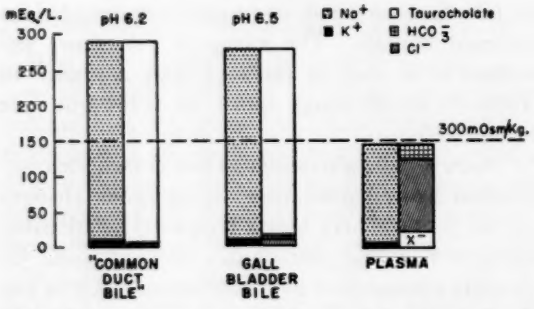

FIG. 1. COMPOSITION OF "COMMON DUCT BILE" COMPARED TO THE COMPOSITION OF CANINE GALL BLADDER BILE AND PLASMA

Bile withdrawn from the common bile duct immediately after catheterization of a fasting cholecystectomized animal (left) had a high taurocholate concentration and very low pH, chloride and bicarbonate. A typical specimen of canine gall bladder bile (center) had a similar composition. In contrast to plasma (right), the sum of the anion and cation concentrations in bile greatly exceeded the osmolality which was close to 300 mOsm. per Kg. in all three solutions.

G. Effect of intravenous secretin

Within five minutes after the intravenous administration of 100 units of secretin a marked choleresis was observed in each dog, exceeding in magnitude the highest spontaneous bile flows observed at any time in the same animal. The duration of the effect was approximately 30 minutes, but the peak of the choleresis was so brief that it was sometimes impossible to obtain adequate specimens of bile representative of the highest flows achieved with this agent. Nevertheless, as shown in Table I and Figure 2 very high values of bicarbonate (up to 59.8 mEq. per L.) and pH (up to 7.78) were seen in bile specimens obtained during secretin choleresis. A typical study in

which secretin was administered is shown in Figure 3. Amylase was not detectable in undiluted bile samples obtained during secretin choleresis although samples of duodenal juice obtained at the same time gave a positive test in dilutions (both in water and bile) as high as 32- to 512-fold.

H. Effect of intraduodenal hydrochloric acid

In order to stimulate the production of endogenous secretin 0.05 N hydrochloric acid was infused into the duodenum (19). A sustained choleresis was observed which was accompanied by changes in composition similar to those seen after exogenous secretin (Table I and Figure 2). Amylase was not detectable in this bile. Bile flow diminished rapidly within 20 minutes after stopping hydrochloric acid administration.

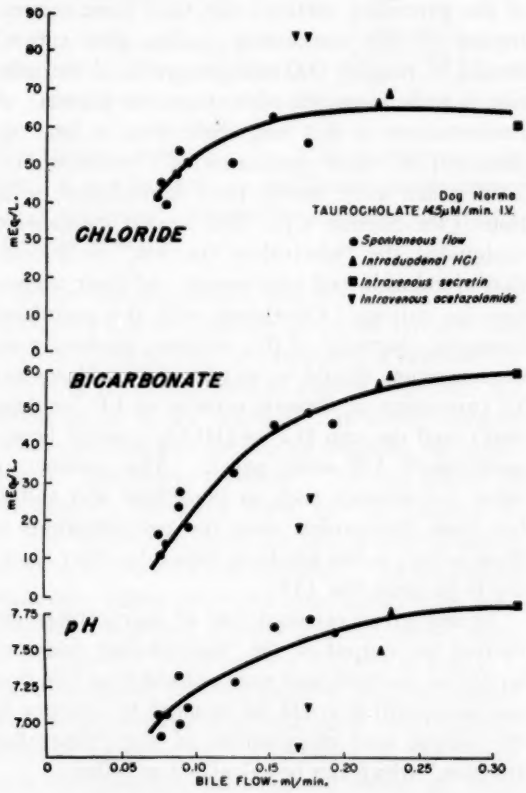

FIG. 2. RELATIONSHIP BETWEEN BILE FLOW AND COMPOSITION

Bile samples were all obtained at least one hour after starting intravenous sodium taurocholate infusion at 14.5 μMoles per minute. Spontaneous variations in flow were apparent and the highest flows were associated with the highest concentrations of bicarbonate and the highest pH. Maximum flow, pH and bicarbonate concentration were observed after intravenous secretin or intraduodenal HCl. After intravenous acetazolamide the chloride concentration was higher and the bicarbonate concentration lower than at comparable spontaneous flows.

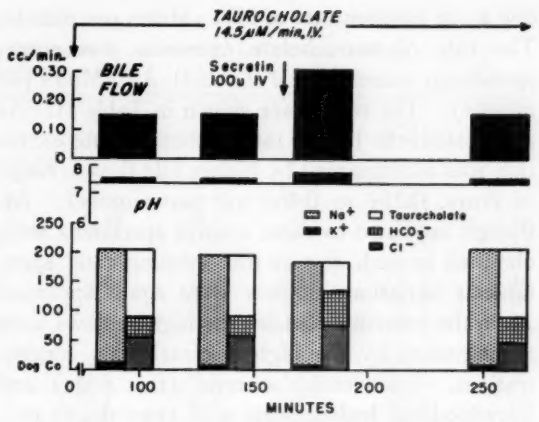

FIG. 3. EFFECT OF INTRAVENOUS SECRETIN

During the course of continuous sodium taurocholate infusion the administration of secretin produced an increase in bile flow. Taurocholate concentration was correspondingly reduced, chloride concentration increased and there was a more marked increase in bicarbonate concentration and pH.

I. Effect of acetazolamide

The intravenous administration of acetazolamide sodium (Diamox®, 65 mg. per Kg.) was followed in each case by a prompt choleresis. In all but one dog the bile flow after acetazolamide exceeded the highest spontaneous flows observed in the same animal. The excretion rate of taurocholate was unaffected. In every case the chloride concentration was higher and the bicarbonate concentration and pH were lower than those observed at similar bile flows under other circumstances (Table I and Figure 2). Thus, in the choleresis produced by this agent, increased excretion of chloride is the predominant feature. In all dogs the choleretic effect was still apparent by the time the study was concluded 60 to 90 minutes after acetazolamide injection.

J. Effect of higher rates of taurocholate infusion

In the foregoing studies sodium taurocholate was infused at a rate of 14.5 μMoles per minute. For comparison one study was conducted on each

dog at an infusion rate of 38.4 μMoles per minute. The rate of taurocholate excretion was correspondingly increased (37.0 ± S.D. 3.1 μMoles per minute). The results are shown in Table II. As anticipated, the higher rate of taurocholate excretion was accompanied by higher bile flows (range of flows, 0.214 to 0.460 ml. per minute). Although only two to three control specimens were obtained in each dog at this infusion rate, spontaneous variations in flow were again apparent. As in the preceding studies the highest flows were accompanied by the highest bicarbonate concentrations. Intravenous secretin (two dogs) and intraduodenal hydrochloric acid (two dogs) produced an increase in flow which appeared, if anything, to exceed the choleretic response produced by these agents at the lower rate of taurocholate infusion. Marked elevations in bicarbonate concentration and pH were again apparent during this choleresis (Table II).

DISCUSSION

These observations re-emphasize the marked dependence of bile production upon the rate of secretion of bile salts, and indicate the necessity for stabilization of this factor if other mechanisms are to be explored. When bile salt excretion rate is constant, a consistent relationship becomes apparent between bile flow and bile electrolyte composition.

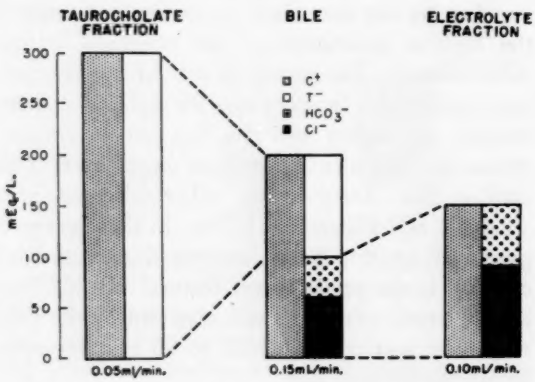

FIG. 4. RELATIONSHIP BETWEEN THE HYPOTHETICAL "TAUROCHOLATE FRACTION" AND "ELECTROLYTE FRACTION" IN A SAMPLE OF BILE

The concentrations of solute in the two fractions are calculated on the assumption that each fraction is isosmotic, as indicated in the text.

The observed variations in flow and composition could be explained by the hypothesis that hepatic bile is formed by admixture of a number of solutions which differ from one another in composition and mode of production. With the limited evidence at hand, it appears reasonable for the sake of simplicity to regard each bile specimen as a mixture of two hypothetical isosmotic components (Figure 4). One of these components, a pure solution of taurocholate and its accompanying cations, has been designated the "taurocholate fraction." In view of the apparent osmotic inactivity of the taurocholate ion itself (see Part D of the preceding section) the total ionic concentration of this component (anion plus cation) should be roughly 600 mMoles per L. if the solution is to be isosmotic with respect to plasma. A concentration of this magnitude was, in fact, approached in some specimens of "common duct bile" which were nearly pure taurocholate solutions (see Section C). The second component, designated the "electrolyte fraction," is a solution of chloride and bicarbonate and their accompanying cations. Consistent with the postulated isosmotic character of this solution, its total ionic concentration should be roughly 300 mMoles per L. (assuming an osmotic activity of 1.0 for these ions), and the sum $[Cl^-]+[HCO_3^-]$ would be approximately 150 mEq. per L. The presence of other constituents such as phosphate and sulfate has been disregarded since the concentrations of these anions in bile has been shown by other workers to be quite low (14).

At any given constant rate of taurocholate excretion the output of the "taurocholate fraction" would be constant, and variations in total bile flow and composition could be equated to changes in the output and composition of the "electrolyte fraction," which can be calculated as follows:

$$V_E = V \cdot \frac{[Cl^-] + [HCO_3^-]}{150}$$

$$[Cl^-]_E = \frac{V}{V_E} \cdot [Cl^-]$$

$$[HCO_3^-]_E = \frac{V}{V_E} \cdot [HCO_3^-]$$

where: $[Cl^-]$ and $[HCO_3^-]$ = chloride and bicarbonate concentrations in whole bile in milliequivalents per liter; $[Cl^-]_E$ and $[HCO_3^-]_E$ = chloride and bicarbonate concentrations in

TABLE II

Relationship between bile flow and electrolyte composition during intravenous sodium taurocholate infusion at 38.4 μMoles per minute

Dog	Condition	Bile flow	Na^+	K^+	HCO_3^-	Cl^-	Taurocholate	pH
		ml./min.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	
	Spontaneous flow	0.221 (min.)	217.8	7.6	21.9	49.1	154.4	7.32
		0.369 (max.)	191.6	6.85	46.4	60.9	91.1	7.59
Norma	Intraduodenal HCl	0.550	182.0	5.85	51.4	72.5	64.0	7.75
	Spontaneous flow	0.300 (min.)	200	7.5	30.2	56.0	121.2	7.46
		0.373 (max.)	197	6.6	40.2	63.6	99.8	7.60
Cora	Secretin I.V.	0.572	191.4	6.6	56.6	80.6	90.6	7.76
	Spontaneous flow	0.214 (min.)	230.4	9.9	12.2	41.3	186.8	6.97
		0.300 (max.)	200.0	11.9	27.9	57.2	126.8	7.49
[illegible]ali	Secretin I.V.	0.733	202.8	8.1	43.8	64.5	102.6	7.70
	Spontaneous flow	0.347 (min.)	197.6	6.2	11.2	77.6	115.0	7.13
		0.460 (max.)	201.2	5.85	20.1	84.1	92.9	7.10
Isolde	Intraduodenal HCl	0.560	202.4	6.0	38.8	94.7	74.9	7.68

"electrolyte fraction" in milliequivalents per liter; V = bile flow in milliliters per minute; V_E = output of "electrolyte fraction" in milliliters per minute.

When this calculation was applied to all bile specimens obtained during constant taurocholate infusion (14.5 μMoles per minute), a relationship similar to that illustrated in Figure 5 became apparent in all of the animals. At lower rates of output chloride was the major anion in the hypothetical "electrolyte fraction." As the output of this fraction increased there was an increase in its bicarbonate concentration and a corresponding decrease in chloride concentration. At maximum choleresis (after secretin) the bicarbonate concentration achieved a value approximately equal to the chloride concentration (75 mEq. per L.). The relationship between chloride and bicarbonate after acetazolamide administration was entirely different (Figure 5). The effect of this agent could be equated to an increased output of "electrolyte fraction" in which chloride was the predominant anion.

The knowledge that bile production is dependent upon the rate of excretion of bile salts, and the observed relationship between flow and electrolyte composition at any constant rate of bile salt excretion are consistent with the view that at least the following processes are involved in normal bile formation:

1) The active secretion of a large quantity of relatively nondiffusible solute (bile salts) into the canalicular system would favor the addition, by passive diffusion, of a volume of water sufficient to maintain normal osmolality. Evidence for such a mechanism has been summarized in a recent re-

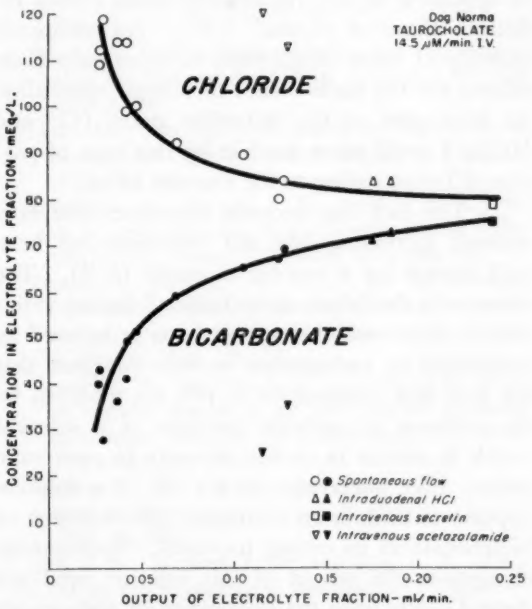

FIG. 5. CONCENTRATIONS OF CHLORIDE AND BICARBONATE RELATED TO THE OUTPUT OF THE HYPOTHETICAL "ELECTROLYTE FRACTION"

Bicarbonate concentration increases and chloride decreases during spontaneous or secretin-induced choleresis. After acetazolamide, there is a preponderance of chloride and correspondingly less bicarbonate.

view by Sperber (20). It must be emphasized that the actual osmolality of canalicular bile is unknown and cannot necessarily be deduced from the observed osmolality of hepatic bile. Nevertheless, it does not seem necessary to invoke a specific mechanism for active water transport, and the likelihood of such a mechanism has been questioned on theoretical grounds (21). The passive diffusion of water in response to active bile salt secretion (and probably, in addition, in response to active secretion of other constituents in lesser amounts) would provide an adequate explanation for the choleretic effect of these substances, and would also be consistent with the observation that bile secretion can continue even when opposed by an appreciable hydrostatic pressure (22). In addition, as a part of this process, concentration gradients would be established which would favor the movement of other solutes into the bile by passive diffusion. Among the latter solutes would be that group of substances which Cook, Lawler, Calvin and Green have referred to as "unconcentrated" (23) and which Brauer has characterized as appearing in bile "in concentrations closely reflecting those of plasma" (24). An additional quantity of water would have to accompany these solutes for the maintenance of normal osmolality. At least part of the diffusible anion (Cl^- and HCO_3^-) could enter the bile by this type of passive diffusion owing to the Donnan effect.

2) The fact that secretin stimulates bile flow without increasing bile salt excretion has been well known for a number of years (6–8). The changes in the "electrolyte fraction" during variations in flow—whether spontaneous or induced by exogenous or endogenous secretin—suggest that the flow and composition of bile are modified by the addition of variable amounts of a solution which is similar in certain respects to pancreatic juice. Like pancreatic juice (25), this solution appears to contain an increasing concentration of bicarbonate as its output increases. Spontaneous changes in the output of this solution need not depend solely upon the elaboration of endogenous secretin. Like the output of pancreatic juice, bile flow has also been shown to vary in response to vagus and splanchnic nerve stimulation (4, 5) so that neurogenic factors may play an important role. The anatomical site of this mechanism has not been established. Although these observations were based on collections of bile through a catheter advanced well into the common bile duct, the possibility of accidental contamination with pancreatic juice was carefully considered. The absence of detectable concentrations of amylase in the bile appeared to rule this out. Moreover, high biliary concentrations of bicarbonate (45.8, 61.2 and 40.5 mEq. per L.) have also been observed after secretin administration during three acute studies in which bile was collected through a catheter tied into the proximal end of the transected common bile duct.

In addition to the two mechanisms discussed above, it is also probable that reabsorption of various constituents may play a major role in determining the final flow and composition of bile. Reabsorption of electrolytes and water is a well established function of the gall bladder mucosa (26, 27). The similarity between the composition of "common duct bile" (see Section C) and gall bladder bile suggests that other parts of the biliary tract may possess similar reabsorptive mechanisms. Whether these are normally operative or whether they tend to "take over" only after cholecystectomy cannot be deduced from the present studies. Considering the high concentration of apparently nondiffusible anion in the bile, these mechanisms would necessarily involve active work against an appreciable oncotic gradient. At present, the actual evidence for reabsorption cannot be considered conclusive.

The choleresis induced by acetazolamide is difficult to correlate with the known pharmacologic activity of this agent. In view of the inhibitory effect of acetazolamide on the volume of pancreatic secretion (28) one would anticipate that this drug would, if anything, depress those processes in the biliary tract which normally elaborate a bicarbonate-rich fluid analogous to pancreatic juice. The fact that bicarbonate concentration during acetazolamide choleresis is lower than that observed during spontaneous or secretin-induced choleresis is therefore not surprising. However, the marked increase in flow and electrolyte excretion implies an altogether separate mechanism involving either augmentation of the movement of electrolytes into the biliary tract or inhibition of their reabsorption. The available data do not permit a choice between these alternatives nor is it easy to see how either could be attributed to carbonic

anhydrase inhibition. The biliary concentration of acetazolamide itself has also not been determined since there are no satisfactory chemical methods available for this purpose. Previous workers have not observed any pharmacologic effect of acetazolamide on bile flow or composition in the rat (29) or man (30). However, in addition to species differences, the experimental circumstances in these instances were entirely different from those reported here.

It must be re-emphasized that bile production in the normal animal varies markedly in response to the demands of the organism. In the species in which it is present, the gall bladder participates in this process by storage, modification and intermittent release of the bile into the duodenum. Enterohepatic circulation of bile salts—the major normal stimulus for bile production—is therefore intermittent in character. Even in the absence of a gall bladder the common bile duct and its major radicles appear to subserve in a similar fashion. The "steady state" which one seeks to achieve by removal of the gall bladder, continuous emptying of the common bile duct and stabilization of the rate of bile salt secretion is therefore, in a sense, not "physiological." Nevertheless, it is possible, by means of this approach, to explore some of the specific mechanisms which are undoubtedly operative in normal bile production.

SUMMARY

The electrolyte composition of bile was studied in four unanesthetized cholecystectomized dogs. The biliary concentration of taurocholate was approximately equal over a wide range to the anion-cation difference: $[(Na^+ + K^+) - (Cl^- + HCO_3^-)]$. The osmolality of all bile samples (mean, 299 mOsm. per Kg.) was indistinguishable from plasma osmolality (mean, 303) although the sum of the electrolyte concentrations in bile greatly exceeded 300 mMoles per L.—a discrepancy probably attributable to the aggregation of taurocholate ions.

Bile stored in the common bile duct of fasting dogs was similar to typical gall bladder bile (high concentration of taurocholate and low concentration of other anions) suggesting the possibility of reabsorptive mechanisms in the bile ducts.

Bile flow was dependent upon the availability of bile salt. When this factor was stabilized by constant infusions of sodium taurocholate, spontaneous variations in bile flow were observed in which high flows were accompanied by high bicarbonate concentration (maximum, 50.9 mEq. per L.) and pH (maximum, 7.81). Highest flows and bicarbonate concentrations were achieved with intravenous secretin or intraduodenal HCl.

Intravenous acetazolamide produced a choleresis in which increased excretion of sodium chloride predominated.

The data suggest that bile production is dependent upon the active secretion of bile salts, and that the flow and composition of bile is further modified by the addition of a solution similar in electrolyte composition to pancreatic juice.

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ACID EXCRETION IN RUBIDIUM- AND CESIUM-SUBSTITUTED RATS * †

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Previous studies have shown that rubidium or cesium can replace a major part of intracellular cation *in vivo*, and can correct the extracellular alkalosis associated with potassium depletion (1). In nephrectomized potassium-deficient animals it was demonstrated that rubidium, like potassium, lowers extracellular bicarbonate concentration by displacement of intracellular protons (2) [the latter perhaps transported out of cells by cationic amino acids (3)]. However, clear quantitative differences can be demonstrated between the effects of rubidium and potassium on extracellular bicarbonate: *a*) chronic administration of RbCl to potassium-deficient alkalotic rats results in extracellular *acidosis*, whereas an equivalent amount of KCl merely restores extracellular bicarbonate concentration to normal (2); and *b*) acute loading of normal rats with RbCl produces a much greater reduction in extracellular bicarbonate than does an equivalent load of KCl (4).

A remarkable feature of the extracellular acidosis produced in normal rats by acute loading with RbCl is the absence of any immediate renal compensation (4). Despite reduction of plasma CO_2 content to 13 mMoles per L. there was no reduction in urine pH, and a slight fall, rather than an increase, in ammonium excretion. Since the administration of acid loads to rats ordinarily results in immediate and very large increments in acid excretion (5–7), the present study was undertaken to learn more about this apparently anomalous behavior of the rubidium-loaded kidney. Although loading with cesium does not produce significant extracellular acidosis, it was of interest to study the acid excretion of the cesium-substituted rat as well, and to compare both the cesium and rubidium preparations with the behavior of the normal and the potassium-depleted animal.

METHODS AND MATERIALS

Male white rats of the Sprague-Dawley strain weighing 200 to 400 Gm. were used in these studies. The animals were divided into four groups. The controls (Group I) consisted of 20 animals fed a low potassium diet for 17 days and then allowed to restore their deficits during a second 17 day period in which they received 20 mEq. per L. of KCl in their drinking water plus the basic diet. There were in addition 20 animals continuously fed on Purina® Chow and never depleted of potassium. Plasma electrolytes were the same in both these subgroups and their subsequent behavior in response to sodium chloride and ammonium chloride loads was indistinguishable. For the purpose of this study, therefore, the depleted-and-then-repleted animals were considered as normal and were grouped together with the Purina®-fed animals, making a total of 40 animals in Group I. Group II consisted of 28 potassium-deficient animals studied immediately after 17 days on the potassium-free diet. Group III was composed of 26 rats which were fed the standard potassium-free diet for 17 days and then given RbCl, 20 mEq. per L., in their drinking water *ad libitum* for the next 17 days. The 30 animals in Group IV were similarly handled, but received 20 mEq. per L. of CsCl in their drinking water.

The plan of the experiment was to observe the 24 hour excretion of acid in each of these groups, under two conditions: *a*) during the administration of NaCl, and *b*) during the administration of an equivalent amount of NH_4Cl. Following the preparatory period of feeding described above, each of the four groups was randomly divided approximately in half, one-half receiving an intraperitoneal load of ammonium chloride solution (1 mEq. per 100 Gm. of body weight, in six equal doses about an hour apart), and the other an equivalent amount of sodium chloride. The loading salts were given at a concentration of 250 mEq. per L. Animals were kept in individual metabolic cages during the 24 hour period of study and all urine was collected under mineral

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TABLE I

Plasma and urine electrolytes

	Plasma *mMoles/L.*				Urine *mEq./day/100 Gm. body weight*					
	CO_2 content		Potassium		Sodium		Potassium		Chloride	
	NaCl	NH_4Cl	NaCl	NH_4Cl	NaCl	NH_4Cl	NaCl	NH_4Cl	NaCl	NH_4Cl
Group I (Normal)	27.2 ± 1.8 (20)	25.6 ± 1.8 (20)	3.6 ± 0.4 (17)	3.4 ± 0.4 (18)	1.18 ± 0.08 (4)	0.31 ± 0.10 (3)	0.34 ± 0.19 (6)	0.38 ± 0.18 (3)	1.11 ± 0.04 (8)	1.16 ± 0.12 (10)
Group II (K-deficient)	32.8 ± 3.0 (15)	29.5 ± 2.9 (13)	2.6 ± 0.4 (15)	2.7 ± 0.4 (11)	0.81 ± 0.05 (3)	0.25 ± 0.11 (3)	0.05 ± 0.02 (3)	0.06 ± 0.10 (3)	1.04 ± 0.10 (6)	1.03 ± 0.12 (6)
Group III (Rb)	19.5 ± 2.8 (13)	16.7 ± 2.3 (13)			1.0 ± 0.10 (3)	0.31 ± 0.03 (3)	0.10 ± 0.01 (3) Rb = 0.02 ± 0.01 (3)	0.19 ± 0.04 (3) Rb = 0.06 ± 0.03 (3)	1.02 ± 0.10 (6)	1.07 ± 0.17 (7)
Group IV (Cs)	26.3 ± 2.7 (16)	19.7 ± 3.8 (14)	1.9 ± 0.3 (11) Cs = 0.43 ± 0.07 (11)	1.9 ± 0.1 (11) Cs = 0.40 ± 0.07 (11)	1.17 ± 0.14 (3)	0.30 ± 0.10 (3)	0.06 ± 0.01 (3) Cs = 0.03 ± 0.10 (3)	0.06 ± 0.01 (3) Cs = 0.03 ± 0.01 (3)	0.92 ± 0.22 (7)	0.87 ± 0.18 (6)

oil using thymol and phenyl mercuric nitrate as preservatives. Animals were deprived of food beginning about 18 hours prior to the day of study and continuing through the remainder of the experiment, but they were allowed unlimited access to drinking water containing 10 per cent glucose.

At the end of the 24 hour study period (about 15 hours after the last dose of salt) the cages were washed down with 10 cc. of distilled water and the washings added to the urine collection. The animals were anesthetized with Amytal Sodium® and exsanguinated from the abdominal aorta. Blood was withdrawn anaerobically into heparinized syringes and the plasma immediately separated for manometric analysis of total CO_2 content. In addition, potassium concentration in the plasma was determined by flame photometry in all animals of Groups I, II and IV. Twenty-four hour urine collections were analyzed for: *1*) pH (glass electrode at room temperature), *2*) titratable acidity (titration with standard alkali, undiluted, using a glass electrode), *3*) CO_2 content (standard manometric technique), and *4*) ammonia (Conway microdiffusion technique). In addition, approximately half of the urines from each subgroup were randomly selected for chloride analysis (potentiometric titration) and, where appropriate, smaller numbers of urines were analyzed for sodium, potassium or cesium (all by flame photometry) or for rubidium (flame photometry after paper chromatographic separation from potassium).

Homogenates of single whole kidneys removed from a number of animals in each of the four NaCl-loaded subgroups were analyzed for glutaminase I activity by the method described by Richterich and Goldstein (8). In addition, single whole kidneys from three randomly selected rats in each of these groups were also analyzed for sodium and potassium content and, where appropriate, for rubidium or cesium. Tissue was minced, extracted with hot water and then analyzed by techniques similar to those applied to urine.

RESULTS

A. Plasma and urine electrolytes

Table I lists the results of the analyses of plasma for potassium and CO_2 content, and also gives the data relating to electrolyte excretion in the urine. For each of the four major groups two sets of values are given. The columns headed "NaCl" list the results for the subgroups loaded with this salt and the columns headed "NH_4Cl" give the data for the subgroups loaded with NH_4Cl. Mean values (± S.D.) are given, and the number of animals is indicated in each case by the figure in parentheses below the data. All urinary excretion data are expressed as mEq. per day per 100 Gm. body weight.

Plasma values. The mean *potassium concentration* was 3.6 ± 0.4 mEq. per L. in the Group I animals loaded with NaCl and was not significantly different[1] in the NH_4Cl subgroup. In both subgroups studied after potassium depletion (Group II) plasma levels were significantly reduced as compared to the normals. Plasma potassium values were not determined in the Group III (RbCl-loaded) animals, but in the CsCl-loaded rats (Group IV) mean potassium concentrations were significantly lower than in the untreated potassium-deficient group.

Plasma *CO_2 content* was normal (27.2 ± 1.8 mMoles per L.) in the NaCl-loaded Group I ani-

[1] Unless otherwise stated, differences between mean values are described as "significant" only when the conventional "t" test yields p values less than 0.01.

TABLE II

Urine pH and acid excretion

	Urine pH		Acid excretion *mEq./day/100 Gm. body weight*					
			Ammonium		Titratable acid		"Net acid"	
	NaCl	NH_4Cl	NaCl	NH_4Cl	NaCl	NH_4Cl	NaCl	NH_4Cl
Group I (Normal)	6.76 ± 0.31 (20)	5.61 ± 0.26 (20)	0.37 ± 0.11 (20)	0.98 ± 0.16 (20)	0.134 ± 0.058 (20)	0.23 ± 0.07 (20)	0.44 ± 0.12 (20)	1.21 ± 0.18 (20)
Group II (K-deficient)	6.47 ± 0.32 (15)	6.13 ± 0.34 (13)	0.55 ± 0.14 (15)	1.30 ± 0.26 (13)	0.092 ± 0.043 (13)	0.18 ± 0.05 (13)	0.62 ± 0.13 (13)	1.46 ± 0.26 (13)
Group III (Rb)	6.20 ± 0.28 (13)	6.04 ± 0.32 (13)	0.32 ± 0.08 (13)	0.98 ± 0.15 (13)	0.107 ± 0.034 (13)	0.13 ± 0.03 (13)	0.40 ± 0.09 (13)	1.08 ± 0.12 (13)
Group IV (Cs)	6.33 ± 0.30 (16)	6.15 ± 0.21 (14)	0.39 ± 0.09 (16)	0.94 ± 0.10 (14)	0.107 ± 0.042 (16)	0.11 ± 0.03 (14)	0.45 ± 0.10 (16)	1.03 ± 0.10 (14)

mals and, as expected, slightly but significantly increased in the similarly treated potassium-deficient animals (32.8 ± 3.0 mMoles per L.). Following the administration of NH_4Cl there was only a slight and insignificant fall in CO_2 content as compared with the respective NaCl subgroups. As will be shown below, this reflected the fact that in both groups most of the acid load was excreted in the urine during the 24 hour period of study. CO_2 content was very low (19.5 ± 2.8 mMoles per L.) in the rubidium-treated animals (Group III) loaded with NaCl but, as in Groups I and II, there was only a small and insignificant further reduction following the NH_4Cl. Plasma CO_2 content in the Group IV (cesium-treated) animals was normal after loading with NaCl (26.3 ± 2.7 mMoles per L.). but was significantly reduced (19.7 ± 3.8 mMoles per L.) after the administration of NH_4Cl.

Urine electrolytes. Chloride excretion ranged from 0.87 to 1.16 mEq. per 100 Gm. body weight during the 24 hour period of study, and there were no significant differences between the various groups, except for a small but significant reduction in the acid-loaded Group IV animals, as compared to the normal. Since the total administered load of NaCl or NH_4Cl was 1.0 mEq. per 100 Gm., it is obvious that in each group of animals essentially all of the chloride load was absorbed and excreted.

Mean *excretion of sodium* in the animals loaded with NaCl ranged from 0.81 to 1.18 mEq. per 100 Gm., demonstrating the virtually complete excretion of the sodium load in all groups. During loading with NH_4Cl excretion of sodium was only 0.25 to 0.31 mEq. per 100 Gm.

Excretion of potassium was 0.34 mEq. per 100 Gm. in the Group I NaCl-loaded animals and, as expected, much lower in the Group II potassium-depleted rats (0.05 mEq. per 100 Gm.). Loading with NH_4Cl did not significantly change these values. In the Group III rubidium-treated animals, excretion of potassium was also lower than in the normal controls (0.1 mEq. per 100 Gm. in the NaCl subgroup, and 0.19 mEq. per 100 Gm. in the NH_4Cl subgroup), and the excretion of rubidium was only 0.02 to 0.06 mEq. per 100 Gm. In the cesium-treated animals, excretion of potassium and of cesium ranged from 0.03 to 0.06 mEq. per 100 Gm.

B. Acid excretion

Table II gives the results of the determinations of urine pH, titratable acidity and ammonium excretion. Also listed is the calculated excretion of "net acid," determined in each animal by the *sum* of titratable acid and ammonium minus any bicarbonate in the urine. Urine bicarbonate, not shown in the table, was very low in the NaCl subgroups and virtually absent in most of the animals loaded with NH_4Cl. The symbols and conventions are the same as those used for Table I.

Urine pH. There were no significant differences between the mean urine pH values of any of the subgroups during sodium loading (range, 6.20 to 6.47), although the mean pH was highest in the potassium-deficient alkalotic animals. During the excretion of the acid load there was a sharp and significant drop in urine pH in the normal (Group I) animals (mean pH 5.61 ± 0.26). There was a somewhat smaller reduction in the potassium-

TABLE III

Increments in excretion of "net acid" and ammonium in response to NH_4Cl (1 mEq./100 Gm.)

	"Net acid"	Ammonium
	mEq./day/100 Gm. body weight	
Group I (Normal)	0.77 ± 0.18 (20)	0.61 ± 0.16 (20)
Group II (K-deficient)	0.84 ± 0.26 (13)	0.75 ± 0.26 (13)
Group III (Rb)	0.68 ± 0.12 (13)	0.66 ± 0.15 (13)
Group IV (Cs)	0.58 ± 0.10 (14)	0.55 ± 0.10 (14)

deficient (Group II) animals (mean pH 6.13 ± 0.34) which was still of significance ($p = 0.01$ to 0.02). However, there were no significant changes with NH_4Cl loading in the rubidium or cesium groups, although in each case the mean urine pH was slightly lower than in the comparable NaCl subgroup.

Ammonium. During NaCl loading, excretion of ammonium in the Group II potassium-deficient animals (0.55 ± 0.14 mEq. per 100 Gm.) was significantly higher than that of any of the other groups, the latter ranging from 0.32 to 0.39 mEq. per 100 Gm. Thus, despite severe systemic acidosis, the ammonium excretion (and urine pH) of the rubidium-treated animals was essentially the same as that found in the normal rats.

In every instance excretion of ammonium was significantly increased during the NH_4Cl load, the increase in the mean for each group ranging from 0.55 mEq. per 100 Gm. in the cesium-treated animals up to 0.75 mEq. per 100 Gm. in the potassium-deficient group.

Titratable acid was relatively low in all groups during NaCl loading (0.092 to 0.134 mEq. per 100 Gm.) and increased significantly during NH_4Cl loading only in the normal and potassium-deficient animals.

Excretion of *"net acid"* during NaCl loading ranged from 0.40 mEq. per 100 Gm. in the rubidium-treated animals up to 0.62 mEq. per 100 Gm. in the potassium-deficient group. The latter group was significantly higher than the normal controls. "Net acid" increased significantly in every case during acid loading, the mean rate of excretion ranging from 1.03 ± 0.10 mEq. per 100 Gm. in the cesium-treated group to 1.46 mEq. per 100 Gm. in the potassium-deficient group.

Table III lists the *increments* in the excretion of "net acid" and ammonium during NH_4Cl loading, as compared to the excretion during NaCl loading. These values were calculated by subtracting the mean excretion in each group during NaCl loading from the excretion of each individual animal during acid loading. The figures shown are the mean values ± S.D. In parentheses is the number of animals in each group. It is seen that the increment in "net acid" ranged from 0.58 mEq. per 100 Gm. in the cesium group, to 0.84 mEq. per 100 Gm. in the potassium-deficient group. The differences in "net acid" between the cesium-treated animals and both the normal and the potassium-deficient animals were significant, but the rubidium-treated animals were not significantly different from any of the other groups. Almost all of the change in "net acid" was due to a change in ammonium excretion, but group differences in the latter were not as large as in "net acid." Only the Groups II and IV showed any significant difference ($p = 0.02$ to 0.05).

C. Kidney tissue analyses

Table IV summarizes the results of the renal tissue analyses. It should be recalled that only kidneys from NaCl-loaded animals were studied. Because of the small number of electrolyte determinations, individual values as well as means are shown. *Potassium content* was slightly but consistently reduced in the three potassium-depleted animals although sodium content was unchanged. In the rubidium-treated animals, potassium was lower than in the untreated Group II rats, while rubidium content was approximately half that of potassium. The cesium-treated animals had the lowest potassium content of all, with a K : Cs ratio of approximately 2.

The *glutaminase* data are given as means ± S.D. There was a significant rise in enzyme activity in all groups, as compared to the normal, the highest activity being found in the rubidium-treated animals.

DISCUSSION

Rats are normally capable of immediate and very large ammonium-excreting responses when their extracellular fluid is acidified by acid loads

TABLE IV

Electrolyte content and glutaminase activity of whole kidneys

	Sodium	Potassium	Rubidium or cesium	Glutaminase
	mEq./100 Gm. dry solids			*μMoles NH_3/min./100 mg. dry solids*
Group I (Normal)	33.0	31.0		18.3 ± 5.1 (11)
	25.5	31.0		
	27.0	32.0		
	Mean 28.5	31.3		
Group II (K-deficient)	26.1	26.2		28.7 ± 9.4 (10)
	29.1	28.6		
	27.6	28.1		
	Mean 27.6	27.6		
Group III (Rb)	25.2	20.5	10.2	57.5 ± 20.7 (9)
	18.4	22.2	12.4	
	22.6	20.5	9.0	
	Mean 22.1	20.9	10.5	
Group IV (Cs)	18.9	14.5	8.3	27.6 ± 9.1 (7)
	21.7	18.3	8.9	
	26.1	16.7	8.9	
	Mean 22.2	16.5	8.7	

(5–7). Nevertheless, despite an extracellular CO_2 content of only 19.5 mMoles per L., the rubidium-treated rats excreted no more acid during loading with NaCl than did the normal animals, thus failing to show any renal response to this disturbance of acid-base balance. The present results would appear to permit certain limited inferences concerning the nature and significance of this phenomenon.

In the first place, it is clear that "competition" between tubular secretion of hydrogen and potassium or rubidium (9) cannot be the explanation for these results. In previous experiments (4), renal acid excretion was measured during the period of acute loading with rubidium, when renal excretion of rubidium and potassium was very high. Such conditions, which might favor inhibition of acid secretion, were avoided in the present experiments by removing rubidium and potassium from the diet prior to and during the 24 hour period of study. Since rubidium is secreted into the urine more slowly than potassium (10), the net result of this maneuver was that the combined secretion of rubidium and potassium was even lower than the excretion of potassium alone in the normal controls (Table I). In none of the groups was the excretion of potassium, rubidium or cesium particularly high.

Secondly, the enzyme assays shown in Table IV demonstrate that the absence of increased ammonium excretion in the rubidium-loaded rats cannot be due to reduced glutaminase activity. As described by other workers (7), the increased ammonium excretion of the potassium-deficient animals was associated with increased glutaminase activity. However, no further correlations could be made between enzyme activity and ammonium excretion because glutaminase was as high, or higher, in the rubidium and cesium groups, *without* increased excretion of ammonium.

Although the rubidium-induced extracellular acidosis does not stimulate acid excretion, the results of the NH_4Cl experiments indicate that rubidium-loaded kidneys are *capable* of normal and prompt acid-excreting responses when given the proper stimulus. As shown in Table III, the increment in "net acid" excretion in response to the NH_4Cl load was virtually the same in the rubidium-treated animals as in the normal or the potassium-depleted groups. A clear implication of these facts is that the administration of NH_4Cl provided a stimulus for the excretion of acid which was otherwise lacking in the rubidium-treated rats, despite their moderately severe extracellular acidosis. On the other hand, as has been observed before (11), there was a high rate of acid excretion in the potassium-deficient animals despite the presence of extracellular alkalosis. Changes in the kidney itself, which may be independent of the acid-base equilibrium of extracellular fluid, are apparently of prime importance in determining acid excretion. The present experiments do not reveal the nature of this intrarenal regulation, but the data in Table IV show that it is not apt to be related simply to the quantities of alkali metal in renal tissue. Thus, acid excretion was essentially equal in Groups I and IV, despite large group differences in renal content of sodium, potassium and potassium plus cesium.

One reasonable possibility is that renal intracellular acidity is the critical factor determining acid excretion. Intracellular acidosis has been suggested as an explanation of the increased acid secretion of the potassium-depleted kidney (12–14). Conversely, it might also be suggested that in the rubidium-loaded animal intracellular pH is normal or increased, since accumulation of rubidium appears to be associated with the movement of acid *out* of cells (2, 4). Renal tissue pH may

fall only when the addition of a large exogenous acid load results in a net movement of acid back into cells. However, little or nothing is known at present about renal cell pH under any *in vivo* conditions. It is obvious that no conclusions can be drawn until there is more information about renal tissue composition and the internal distribution of acid in potassium-depleted and rubidium- and cesium-substituted animals.

A final topic of interest is the defect in acidification of the urine which was demonstrated in both the rubidium- and cesium-treated animals. In the normal rats, administration of NH_4Cl sharply reduced urine pH coincidentally with the increased excretion of ammonium (Table II). In the rubidium- and cesium-treated groups, although the ammonium response was normal, there was only a very slight and insignificant lowering of urine pH (Table II). Leonard and Orloff have demonstrated that ammonium is not as closely related to urine pH in the normal rat as in dog or man (5). The present observations show that it is possible to produce virtually independent changes in these functions, thus suggesting that establishment of final hydrogen-ion gradients may be an operation which is biochemically or anatomically separate from the ammonium-excreting mechanism. Loss of potassium from cells and substitution of rubidium or cesium in some manner impairs the hydrogen ion gradient process without inhibiting the capacity to excrete ammonium. In untreated potassium depletion, the relative alkalinity of the glomerular filtrate may be, at least in part, responsible for the alkalinity of the urine (11), but this factor clearly cannot be implicated in the rubidium-substituted rats in which the plasma bicarbonate is markedly reduced.

SUMMARY AND CONCLUSIONS

To learn more about the renal responses of rats loaded with rubidium or cesium, experiments were designed to compare acid and electrolyte excretion during a 24 hour period in: *a*) normal animals, *b*) potassium-depleted alkalotic animals, *c*) rubidium-substituted animals, and *d*) cesium-substituted animals. These groups were compared during administration of NaCl and also during loading with NH_4Cl.

During NaCl loading, ammonium excretion was higher than normal in the potassium-deficient animals, but not increased in those treated with cesium or rubidium, despite a significant degree of extracellular acidosis in the latter group. Glutaminase activity was increased in the potassium-deficient animals as well as in those treated with rubidium and cesium. Excretion of potassium (or rubidium or cesium) was minimal and renal potassium content was low in all the experimental groups.

All the animals responded to the NH_4Cl load with a large and approximately equivalent increment in ammonium excretion. However, in contrast to the sharp drop in urine pH in the normal animals, there was no significant change in the acidity of the urine in the rubidium- or cesium-treated groups.

It is thus demonstrated that rubidium-substituted kidneys do not respond to the extracellular acidosis produced by the rubidium, but are nevertheless capable of a normal ammonium response to the administration of an acidifying salt. The data are consistent with the hypothesis that the normal stimulus to ammonium excretion is related to intracellular rather than extracellular acid-base equilibrium. It is tentatively suggested that renal intracellular pH may be normal or increased in the rubidium-substituted animal.

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PROGESTERONE EFFECTS ON GALACTOSE METABOLISM IN PREPUBERTAL PATIENTS WITH CONGENITAL GALACTOSEMIA AND IN RATS MAINTAINED ON HIGH GALACTOSE DIETS *

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In a recent publication (1) it was reported that *in vitro* addition of certain steroid hormones causes a 200 to 300 per cent stimulation of galactose-1-C^{14} oxidation to $C^{14}O_2$ by rabbit liver slices. Progesterone, androsterone and testosterone produced maximal stimulatory effects. Further data have been obtained (2) which demonstrate *a*) that of many tissues tested, only liver and intestine respond to the steroids; *b*) the same steroid specific effect can be obtained in a 100,000 × G supernatant fraction of rabbit liver homogenates; and *c*) the *in vitro* steroid stimulation of galactose oxidation is mediated by an indirect stimulation of the uridinediphosphogalactose-4-epimerase (UDPGal-4-epimerase) reaction.

During the course of these investigations some *in vivo* effects of progesterone on galactose oxidation were investigated. The results of progesterone administration to three prepubertal galactosemic subjects and to young rats maintained on high galactose diets serve as the basis for this report.

MATERIALS AND METHODS

Case Histories [1]

Case 1: L.W.J., 11.5 year old white male. The patient was the product of a normal full term pregnancy and delivery. There was no family history of metabolic disorder. At the age of two weeks the patient began to have frequent episodes of vomiting and diarrhea in spite of frequent changes in formula. Because of continued gastrointestinal irritability and failure to gain weight, at age 2.5 months he was admitted to Sinai Hospital in Baltimore where after careful study (3) the diagnosis of congenital galactosemia was made. The patient was placed on a galactose-free diet and when seen again three months later, had gained weight and showed regression of cataracts and improvement of liver function tests. During the succeeding years the patient did quite well except for difficulty with vision and poor performance in school. On admission to the Clinical Center the patient exhibited bilateral nuclear cataracts, slight hepatomegaly and mental retardation with a mental age of seven years and an I.Q. of 59. The hemogram and blood serology were normal. Blood urea nitrogen was 9 mg. per 100 ml., fasting blood glucose 84 mg. per 100 ml., total cholesterol 182 mg. per 100 ml., cephalin flocculation was negative and total serum proteins and albumin to globulin ratio were normal. Urinalysis on several occasions revealed no reducing substance or proteinuria. The hemolysate assay for galactose-1-phosphate uridyl transferase (P-Gal transuridylase) of Anderson, Maxwell and associates (4, 5) substantiated the diagnosis of congenital galactosemia.

Case 2: E.L.W., 11.5 year old white male. This patient was also the product of a normal pregnancy and delivery. There was no family history of metabolic disorder. The mother is known to have discoid lupus erythematosus. The patient manifested no abnormalities in feeding or development during the neonatal period. An injury at the age of three months prompted hospital admission and he was diagnosed as having malnutrition and hepatomegaly. He was empirically placed on a diet of Mulsoy® with elimination of milk and milk products. Addition of milk to this regimen produced vomiting and diarrhea. He was hospitalized again at the age of two years because of pneumonia. At this time he was again noted to have hepatomegaly and showed evidence of mental retardation. He apparently did fairly well until age 10 years when he began to have difficulty with his vision. About 1.5 months prior to his admission to the Clinical Center, a physical examination was arranged at the Johns Hopkins Hospital. Bilateral cataracts were noted and following admission and thorough work-up, including galactose tolerance and the hemolysate assay for P-Gal transuridylase, the diagnosis of congenital galactosemia was made and the patient was placed on a rigid milk-free diet for the first time in his medical his-

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[1] The authors gratefully acknowledge the assistance of Dr. James B. Sidbury, Jr. of Johns Hopkins University in obtaining these patients for study.

tory. The patient was then transferred to the Clinical Center for further study.

Case 3: J.J.O., 7 year old white male. This patient was the product of a normal pregnancy and delivery. Two siblings also have been diagnosed as having congenital galactosemia. There is no family history of other metabolic disorder. Following development of neonatal vomiting, diarrhea, jaundice and failure to gain weight, the patient was hospitalized and the diagnosis of galactosemia was made on the basis of abnormal galactose tolerance. Subsequent confirmation of the diagnosis has been obtained by the hemolysate assay for P-Gal transuridylase. Because of rigid adherence to a completely galactose-free dietary regimen, this patient has done very well, manifesting little clinical evidence of galactosemia.

During the course of these studies all three subjects were maintained on galactose-free diets.

Procedures

Galactosemic studies. Two μc. of galactose-1-C^{14} was administered intravenously following an overnight fast, and samples of expired air were collected in Douglas bags at intervals during the six hour period immediately following administration of the isotope. These samples were analyzed for total CO_2 content by the method of Fredrickson and Ono (6). The volume of expired air was measured using a wet-test gas meter after removal of CO_2 by bubbling through CO_2-free NaOH. Carbonate was collected by precipitation with 20 per cent $BaCl_2$. The resulting $BaCO_3$ was washed with water until the supernatant solution was no longer alkaline, and dried *in vacuo* at 110° C. overnight. CO_2 was liberated from a weighed amount of $BaCO_3$, diffused into hyamine base and specific activity determined by assaying for $C^{14}O_2$ content using a Tricarb[2] Liquid Scintillation Spectrometer (7). The cumulative expiratory excretion of $C^{14}O_2$ was then calculated according to Berlin, Tolbert and Lawrence (8).

Following a control study, as outlined above, Subject 1 received daily 10 mg. intramuscular injections and Subjects 2 and 3 received daily 20 mg. intramuscular injections of an aqueous suspension of progesterone (Syngesterone®, Pfizer) for a period of six days. On the seventh day (24 hours following the last injection of the hormone) an identical $C^{14}O_2$ excretion study was performed. Subject 1 received galactose-1-C^{14} of specific activity 1.76 μc. per mg. and Subjects 2 and 3 received galactose-1-C^{14} of specific activity 4.72 μc. per mg. In all cases galactose of equal specific activity was used in the control and postprogesterone studies. Galactose-1-C^{14} was purchased from the National Bureau of Standards

[2] Packard Instrument Company, LaGrange, Ill.

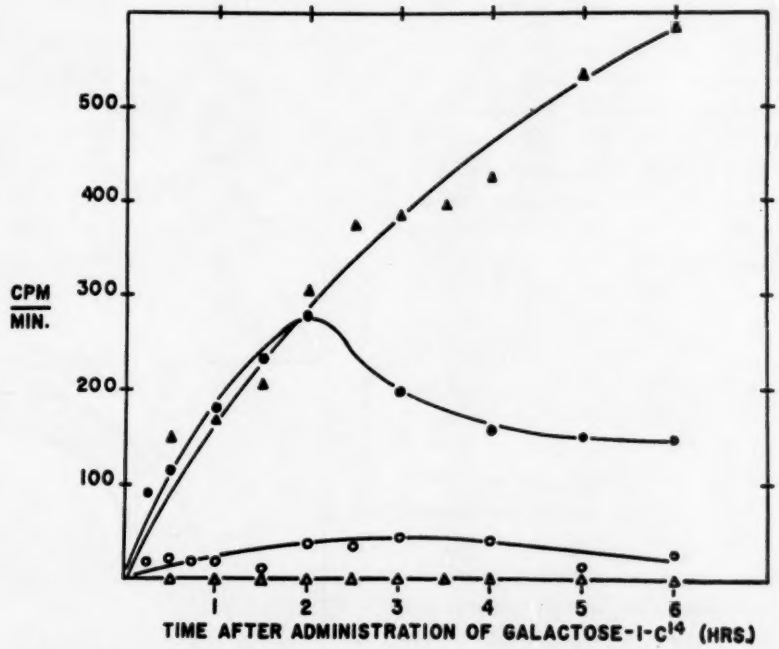

FIG. 1. EXPIRATORY EXCRETION OF $C^{14}O_2$ BY GALACTOSEMIC PATIENTS AFTER PROGESTERONE ADMINISTRATION

Subject 1, L.W.J., (○) before progesterone administration; (●) after progesterone administration.

Subject 2, E.L.W., (△) before progesterone administration; (▲) after progesterone administration.

and was sterilized as a saline solution by filtration prior to use.

Cataract studies. Young Sprague-Dawley male rats weighing 70 to 80 Gm. for galactose and 30 Gm. for xylose experiments were randomly divided into groups of 10 and maintained *ad libitum* on a 30 or 40 per cent galactose diet or a 30 per cent xylose diet prepared by adding the carbohydrate weight for weight to finely ground Purina® Rat Chow. An aqueous suspension of progesterone (Syngesterone®, Pfizer) was administered daily to the treated groups by intramuscular injection of 2.5 mg. per animal. Animals were observed daily for cataract development. Cataracts were counted only when nuclear opacification was complete and clearly visible to the unaided eye. Cumulative incidence of cataract formation was followed rather than animal incidence making a total of 20 possible cataracts in each group of 10 rats. D-galactose and D-xylose were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio or Pfanstiehl Chemical Company, Waukegan, Illinois.

RESULTS

Effect of progesterone administration on the expiratory excretion of $C^{14}O_2$ by galactosemic subjects following administration of galactose-1-C^{14}

The expiratory excretion of $C^{14}O_2$ following intravenous injection of galactose-1-C^{14} before and after progesterone administration is shown in Figure 1. During the control period in both cases, no significant amount of $C^{14}O_2$ could be detected in the expired air. Following six days of progesterone administration a significant amount of the administered isotope appeared in the expired air. The calculated cumulative excretion during the six hour collection period following treatment is 7 per cent of the injected C^{14} for Subject 1 and 12 per cent for Subject 2. The third patient studied in this manner is not shown on this graph because the study was not carried out for the complete six hour collection period. However, the results obtained during a two hour control period and a four hour post-treatment period are similar to the results obtained from Subject 2. Since the results are based on samples containing relatively small amounts of radioactivity, samples of red cell hemolysates were analyzed for galactose-1-phosphate[8] (gal-1-P) to see if the increase in excretion observed after progesterone administration was due to falling levels of gal-1-P following hospitalization and institution of a strict galactose-free diet. Within the limits of the method (9) the erythrocytic gal-1-P levels did not change between the control and post-treatment isotope studies. The difference in the shape of the two post-treatment excretion curves cannot be explained at this time but may be related to the fact that different absolute amounts of galactose were administered to the two patients and the dosage of progesterone was different in the two experiments. Furthermore, since the post-treatment excretion curve of Subject 2 is incomplete, the ultimate excretion of $C^{14}O_2$ should be much greater than the 12 per cent observed during the first six hours.

It would be of interest to know what the nongalactosemic prepubertal excretion curve is following administration of galactose-1-C^{14}. However we have not been able to justify performance of this study because of the ethical implications attendant to the administration of radioactive isotopes to children when such studies are not directly related to diagnosis or possible therapy.

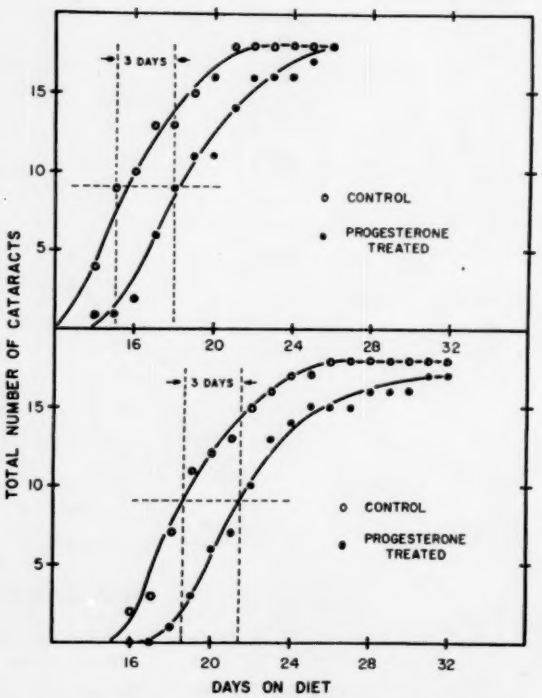

FIG. 2. EFFECT OF PROGESTERONE ADMINISTRATION ON CUMULATIVE INCIDENCE OF CATARACTS IN RATS MAINTAINED ON 40 PER CENT GALACTOSE DIETS

[8] We are indebted to Dr. H. N. Kirkman of the National Institute of Arthritis and Metabolic Diseases who kindly carried out these determinations.

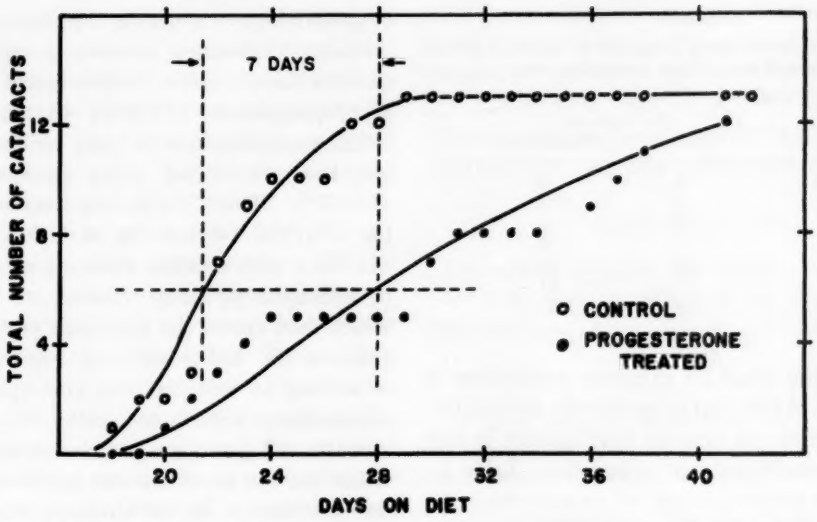

FIG. 3. EFFECT OF PROGESTERONE ADMINISTRATION ON CUMULATIVE INCIDENCE OF CATARACTS IN RATS MAINTAINED ON 30 PER CENT GALACTOSE DIETS

Effect of progesterone administration on cumulative incidence of cataract formation in rats maintained on high galactose and xylose diets

The results of two replicate experiments using a 40 per cent galactose diet are shown in Figure 2. It can be seen that the total number of cataracts was the same in both the control and treated groups. However, there is a three day delay in the time taken for half the total number of cataracts to develop in the progesterone-treated group. In an attempt to accentuate this difference, an experiment was conducted using a 30 per cent galactose diet. These results are shown in Figure 3. Using this lower galactose content diet, the *total* number of cataracts was less and again it can be seen that the same number of cataracts ultimately developed in both groups. However, the time for development of half the total number of cataracts is now delayed seven days by treatment with progesterone.

Another parameter of the progesterone effect on cataract formation is shown in Figure 4. Daily incidence of cataract development is plotted against day of the cataract development period. Thus, day 1 of this period becames the day of observance of the first cataract in all experiments independent of the dietary regimen. Not only is there a significant delay in the peak incidence but there is also a decrease in the daily incidence of cataract development, $t = 2.50$, $p < 0.02$. This is more apparent in the group on the 30 per cent galactose diet. No differences were observed between control and treated groups with regard to weight gain, dietary consumption or general appearance. That

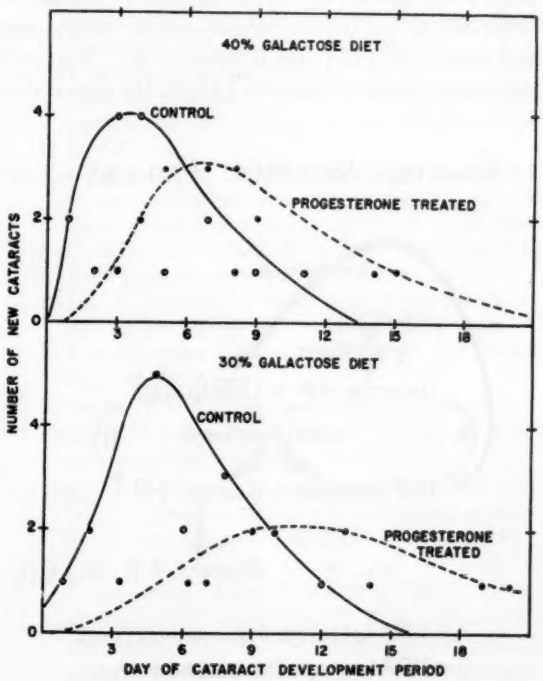

FIG. 4. EFFECT OF PROGESTERONE ADMINISTRATION ON DAILY INCIDENCE OF CATARACTS IN RATS MAINTAINED ON 30 AND 40 PER CENT GALACTOSE DIETS

TABLE I

Effect of progesterone on daily incidence of cataracts in rats maintained on a 30 per cent xylose diet

Days on diet	No. cataracts	
	Control	Progesterone
1–6	0	0
7	5	6
8	8	8
9	8	8
10	10	11
11	10	11
12	10	11

this is a specific effect on galactose metabolism is suggested by the fact that progesterone administration had no effect on cataract development in rats maintained on a 30 per cent xylose diet (Table I).

DISCUSSION

Congenital galactosemia is a disease characterized by the development of cataracts, mental retardation and liver damage (10). These manifestations are thought to be the result of excess intracellular accumulation of gal-1-P (11, 12). The biochemical lesion in this disease is a genetically determined deficiency of the enzyme P-Gal transuridylase as has been demonstrated by Isselbacher and associates (13) and Kalckar (14). Figure 5 depicts the known reactions (15) in the conversion of galactose to CO_2 via the 6-phosphogluconic acid pathway. Galactose is first phosphorylated by galactokinase. Gal-1-P then reacts with uridine diphosphoglucose (UDPG) in the presence of P-Gal transuridylase to yield uridine diphosphogalactose (UDPGal) plus glucose-1-phosphate (G-1-P). The UDPGal is then epimerized by the UDPGal-4-epimerase to UDPG which then can react with another molecule of gal-1-P or be pyrophosphorylytically cleaved to yield G-1-P which then enters the glycolytic pathway. In the galactosemic individual, the transferase enzyme is lacking or deficient and consequently gal-1-P accumulates within the cell. However, small amounts of galactose can be metabolized (16) either via the small amount of P-Gal transuridylase present or via an alternate enzyme recently described by Isselbacher (17). In this latter instance the P-Gal transuridylase deficiency can be circumvented by an additional pyrophosphorylytic reaction. Gal-1-P under these circumstances is converted to UDPGal by the enzyme UDPGal pyrophosphorylase (17) (Step A, Figure 5). UDPGal is then epimerized by the UDPGal-4-epimerase to UDPG which in turn can be converted to G-1-P by UDPG pyrophosphorylase (Step B, Figure 5). The extent to which such an alternate pathway operates in the normal or in the galactosemic individual is not known.

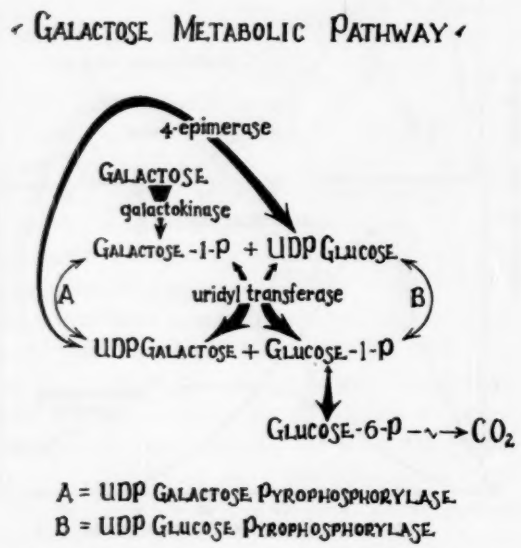

FIG. 5. REACTIONS INVOLVED IN THE CONVERSION OF GALACTOSE TO CO_2 VIA THE PHOSPHOGLUCONIC ACID OXIDATIVE PATHWAY

The mechanism by which progesterone exerts its stimulatory effect on the oxidation of galactose-1-C^{14} to $C^{14}O_2$ in the galactosemic individual is unknown. These studies were conducted before it was known that the UDPGal-4-epimerase reaction, an enzymatic step beyond the metabolic block in galactosemia, is the site of the *in vitro* progesterone stimulation of galactose metabolism (2). The *in vivo* observations may be the result of a completely different type of mechanism such as induction of the deficient or an alternate enzyme. It is therefore apparent that the explanation of this effect must await further experimental evidence.

At the present time there is no good experimental animal in which to study the complete picture of galactosemia other than the human subject. However, one of the parameters of the disease is the development of cataracts and it is a well established observation that high galactose and xylose diets will produce cataracts in young rats

(18–20). This is not meant to imply a relationship between the mechanism of production of cataracts in the two different situations. However, the observation that progesterone administration delays the onset and decreases the *daily* incidence of cataract development in young rats maintained on high galactose diets does indicate that progesterone in some way acts to ameliorate the toxicity of galactose in high concentration. This is supported by the observation that progesterone exerted no effect on the development of cataracts by rats maintained on high xylose diets. Here again a relationship cannot be drawn between the *in vitro* effect of progesterone on the rate of the UDPGal-4-epimerase reaction and the *in vivo* effect on cataract formation. Several attempts were made to study the effect of progesterone on the rate of galactose-1-C^{14} conversion to $C^{14}O_2$ by lens but the rate of oxidation was so low that no reliable data could be obtained.

It is of interest to the interpretation of these results that Schwarz and Golberg (21) have reported increased accumulation of gal-1-P in the optic lens capsule of rats fed high galactose diets. These levels of gal-1-P were similar to those reported to occur in erythrocytes of patients with congenital galactosemia (11).

At the present time it is not possible to ascribe any therapeutic benefit for the galactosemic patient to the administration of progesterone based on the results of these tracer or dietary studies. Such a conclusion must await further careful evaluation. Even under optimal conditions progesterone administration could not be expected to replace the present management of galactosemia. However, progesterone might favorably act to minimize the progression of cataract formation and mental deficiency, especially during the periods of the disease when patients are acutely ill as a result of ingestion of high milk diets, or during exacerbations when cataract development and mental retardation seem to be progressive.

SUMMARY

Administration of progesterone to three prepubertal patients with congenital galactosemia resulted in a significant increase in ability to oxidize a tracer dose of galactose-1-C^{14} to $C^{14}O_2$. Furthermore, administration of progesterone to young male rats maintained on high galactose diets caused a delay in the onset and a decrease in the *daily* incidence of cataract formation. These observations are discussed in light of *in vitro* effects of progesterone and in terms of possible therapeutic implications in congenital galactosemia.

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TRANSFER OF DELAYED HYPERSENSITIVITY TO SKIN HOMOGRAFTS WITH LEUKOCYTE EXTRACTS IN MAN *

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There is general agreement that the rejection of foreign homologous tissues applied as homografts to genetically unrelated individuals is accomplished by an immunological mechanism undertaken by the host (1–5). It is not yet clear, however, whether the highly specific immune mechanism evoked and the consequent allergic inflammatory response resulting in tissue destruction are mediated by serum antibody of the classical type or by a factor or factors intimately bound to cells of the leukocyte series (6–8). The preponderance of evidence accumulated to date has implicated the latter mechanism (9–14), although there is evidence to suggest a role for serum antibody, either as a contributing ancillary agent of tissue destruction (4, 15–17), or as a paralled but unrelated immunological event (1, 18, 19).

Much interest has converged upon the outcome of this issue, since any efforts designed to ameliorate or abolish an immunological response with this high degree of specificity are logically dependent upon the precise definition of the specific antigen or antigens that induce it and the specific antibody or antibodies that mediate it.

The most convincing evidence for a cellular effector mechanism has accrued from the successful transfer of accelerated homograft rejection of tumors by Mitchison (9, 18, 19) and skin by Billingham, Brent and Medawar (10, 11) in mice, and similar transfer of delayed tissue reactivity to homograft antigens in the guinea pig by Brent, Brown and Medawar (12). This application of the cellular transfer system to the study of homograft hypersensitivity in animals has done much to bring the mechanism mediating homograft hypersensitivity in close analogy to that shown earlier by Landsteiner and Chase (20) and by Chase (21) to mediate contact chemical and bacterial hypersensitivities of the delayed type.

In the human subject, the application of the cellular transfer system to the study of bacterial (22–25), fungal (26), viral (27, 28), and contact chemical (25, 29) hypersensitivities of the delayed type has resulted in additional insight into the immunity mechanisms mediating this general type of altered tissue reactivity. The unique feature of this system in man, in contrast to animal species, is the finding that extracts of leukocytes are as effective as intact viable cells in the transfer of delayed hypersensitivity (30–34). This observation has afforded an opportunity to attempt analysis of the mechanisms involved and identification of the factor or factors (transfer factor) in sensitive human leukocytes concerned with the transfer of delayed hypersensitivity. The precise biochemical or immunological nature of transfer factor is not known; however, certain properties of the biologically active principle have been described (35–37).

Briefly, transfer factor obtained from minute quantities of peripheral blood leukocytes (0.01 to 0.5 ml.) endows the recipient with the specific sensitivity (ies) of the donor within a matter of hours; the transferred sensitivity is generalized and may endure for as long as one to two years. The success of transfer is governed by the degree of sensitivity of the donor, the volume of leukocytes used and an unknown contribution of the recipient. Leukocytes from nonsensitive individuals are ineffective in this regard. Transfer factor has been shown unable to cross the species barrier;

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it resists freezing and thawing and treatment with the enzymes desoxyribonuclease (DNA-ase), ribonuclease (RNA-ase) or trypsin. Transfer factor interacts with but is not neutralized by antigen, and conventional antibody is not detectable in donor leukocyte extracts nor in recipient's sera or skin reactivity following transfer; it may be readily liberated from sensitive leukocytes into cell-free supernatant solutions (*22, 23, 26,* 30, 31, 38).

In the present paper, we report on the transfer of homograft hypersensitivity (accelerated homograft rejection) in man by means of an injection of DNA-ase-treated leukocyte extracts obtained from the peripheral blood of specifically sensitized donors.

MATERIALS AND METHODS

Basic plan of experiments

1) Skin grafts from Subject A were used to sensitize Subject B. *2*) Anti-A leukocytes were obtained from B; an extract was prepared, and injected into C. *3*) Sensitivity transferred to C was measured by the rejection period of a *test* graft (skin from A), as compared to that of a *control* graft (skin from D) from a subject unrelated to the experiment. *4*) A positive result was scored when the test graft was rejected in an accelerated fashion (4 to 6 days) while the control graft was rejected as a first set graft (10 to 12 days).

Sensitizing material and method of sensitization

Under local anesthesia the donor site was incised with a circular trephine (11 mm. diameter) and a full-thickness skin graft excised (39). The graft was placed on gauze saturated with normal saline and the undersurface freed of subcutaneous tissue. A graft bed area, identical in size, was prepared in similar fashion in the recipient upon the radial aspect of the volar surface of the forearm. Upon cessation of bleeding and capillary oozing in the recipient bed, the graft was inserted into the defect and its edges approximated to the recipient skin with interrupted (5–0 nylon) sutures. The area was covered with a layer of fine nylon mesh and a pressure dressing applied. Alternate sutures were removed on the third day and the remaining sutures on the fourth day.

Schedule of sensitization of leukocyte donors

Two schedules of leukocyte donor sensitization were employed, differing from each other in the time interval between application of the sensitizing skin grafts. *1*) The second set, third set and fourth set grafts from the same skin donor were applied two to three weeks after the occurrence of accelerated rejection of the preceding skin graft. This resulted in the accelerated rejection of each skin graft applied after the first set; *2*) the second set, third set and fourth set skin grafts were applied seven days after the rejection of the preceding graft. This schedule resulted in the occurrence of a "white graft" reaction (*i.e.,* vascular connections between graft and host did not occur) in each graft applied after the first set (40, 41).

Criteria of homograft rejection

Two methods were used routinely to determine the onset of the rejection period in all skin grafts.

1. Microscopic appearance of graft rejection. The method of stereomicroscopic observation of the graft capillaries devised by Taylor and Lehrfeld (42) and adapted to man has been used throughout this study; it has been described in detail elsewhere (39). With this method, the ultimate macroscopic rejection of the skin graft is predicted by the microscopic changes, which include cessation of capillary blood flow, intracapillary thrombosis and extravasation of blood into the graft. This method was employed from the third postoperative day forward and served also to indicate the onset of blood flow in the capillaries of homografted skin, as a critical measure of satisfactory union between host and graft.

2. Gross appearance of graft rejection. The gross characteristics of graft rejection were regularly preceded by the microscopic findings described above, and consisted of graft cyanosis, hemorrhage and swelling, with an area of surrounding erythema and induration in the recipient skin, the graft ultimately undergoing necrosis and escharification.

The criteria for graft rejection therefore included: *1*) development of erythema and induration around the graft; *2*) cyanosis and edema of the graft; *3*) cessation of blood flow and capillary thrombosis in the graft vessels. The subsequent development of an eschar, and its sloughing, confirmed the diagnosis of graft rejection in each case.

Using these criteria, it had been determined earlier, in an experimental study of the homograft rejection phenomena in human skin (39), that the average time of rejection of first set grafts is 10 to 12 days, whereas a second graft from the same donor to the same recipient, if applied two to three weeks later, will be rejected in an accelerated fashion (four to six days), as a manifestation of actively acquired homograft sensitivity, specifically directed against the tissues of that particular donor (40).

Preparation of leukocyte extracts

Leukocytes were isolated from the peripheral blood of sensitized donors by a modification of the usual technique (22, 23, 30) which has been described in detail elsewhere (26). Briefly, 400 ml. of whole blood was collected from each donor, through a cation-exchange resin chamber, into a plastic Fenwal bag to which was added 100 ml. of a Seitz-filtered distilled water solution containing 0.05 Gm. per ml. bovine fibrinogen (Fraction I, Armour). The contents were mixed, and the bag was suspended in a 37° C. incubator for 30 to 45 minutes; the sedimented erythrocytes were siphoned off, and the plasma supernatant suspension of leukocytes was collected in conical-tip centrifuge tubes graduated in 0.01

TABLE I

Homograft rejection patterns of leukocyte donors sensitized for transfer experiments

Skin donor	Skin recipient WBC donor	Time of rejection of first set grafts	Interval between grafts	Time of rejection of second set grafts	Interval between grafts	Time of rejection of third set grafts	Interval between grafts	Time of rejection of fourth set grafts
		days	*days*	*days*	*days*	*days*	*days*	*days*
Dav	Jos (D1)	15–16	50	4				
Mat	Bow (D2)	18–19	18	5–6				
Mat	Rubn (D3)	13–15	22	6–7				
Mat	Fol (D4)	9–10	27	5–6				
Abr	Jos (D5)	11–12	17	7–10				
Abr	Bar (D6)	11–13	20	7–9				
Cox	Wal (D7)	9	22	4	26	4	22	4
Fol	Jos (D9)	8–10	23	5–6	22	4–5	17	4–5
Jos	Fol (D10)	5–6	28	6–7	21	4	18	5–6
Car	Cas (D8)	7–8	7	0*	7	0*	7	0*

* White graft reaction.

ml., and centrifuged at 2,500 rpm for 15 minutes. The volume of packed leukocytes per tube was recorded, the supernatant plasma pipetted off and the leukocytes aspirated with a Pasteur pipette. The pooled leukocytes were resuspended in sterile, pyrogen-free nomal saline, and complete leukocyte disruption was accomplished by alternate rapid freezing (95 per cent alcohol-dry ice) and thawing (37° C. water bath) for 7 to 10 cycles. The leukocyte extract was treated with a solution containing 1 mg. per ml. crystalline pancreatic desoxyribonuclease (Worthington) in the presence of Mg^{++} before injection into the recipient.

EXPERIMENTAL AND RESULTS

Selection of skin donors and recipients

Skin donors and recipients were selected from a stable population of healthy volunteers who were pedigreed blood donors known not to transmit homologous serum hepatitis. These individuals were either physicians on the house staff of the Third (N. Y. U.) Medical or Surgical Divisions of Bellevue Hospital, or students at the New York University College of Medicine. The recipients of skin grafts were also selected with regard to the above pedigree, since following sensitization to the respective skin grafts, they would in turn become the donors of the leukocytes used for transfer purposes.

Selection of recipients of leukocyte extracts

This group of volunteers was identical to that described above with the exception that there was no necessity for the recipient to be a pedigreed blood donor. In addition to the physicians and medical students who participated in this study, six patients on the Fractures Service also served as recipients of transfers (see Table IV).

Results of sensitization experiments

The responses of each individual who served as a leukocyte donor in the transfer experiments to the initial sensitizing (first set) and subsequent (second set, third set and fourth set) skin grafts from the same source are summarized in Table I.

It may be seen that the onset of the rejection of first set grafts varies from as early as five to six days in one pair of individuals to 18 to 19 days in another pair. The onset of accelerated rejection varies from 7 to 10 days at its latest to four days at its earliest. The average time of onset for the rejection of first set grafts was established to be 8 to 12 days, and four to six days for the accelerated rejection of subsequent grafts. This is based upon earlier (39–41) as well as current observations.

Results of transfer experiments

A. Use of one or of two consecutive skin homografts to sensitize the leukocyte donor.

1. Systemic transfer. The graft was applied at a site remote from the injection of the leukocyte extract.

Systemic transfer was used exclusively in this report, except in the experiments detailed in Section 2 below. It consists of the intradermal and subcutaneous injection of sensitized leukocyte extract into the skin overlying the shoulder of the

TABLE II

*Effects of systemic transfer from leukocyte donor sensitized with one or two grafts**

Leukocyte donor	Sensitization of leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage WBC	Recipient No.	Day of graft rejection: Test graft	Day of graft rejection: Control graft	Interpretation of result
					ml.				
D1	1 Graft	Systemic	13 Days before grafts applied	WBC extract	0.5	R1	11 11	9 9	Negative
						R2	12 12	Poor take 9	Negative
D2	2 Grafts	Systemic (pooled WBC first and second set rejection)	5 Days before grafts applied	WBC extract	1.28	R3	11 11	8 8	Negative
D3	2 Grafts	Systemic (pooled WBC first and second set rejection)	5 Days before grafts applied	WBC extract	1.06	R4	10 10	7 7	Negative

* Skin donor	Skin recipient WBC donor	WBC recipient	Test graft donor	Control graft donor
Dav	Jos (D1)	McK (R1)	Dav	Mar
		Mar (R2)	Dav	McK
Mat	Bow (D2)	Rob (R3)	Mat	Jaf
Mat	Rubn (D3)	Bri (R4)	Mat	Jaf

nonsensitive recipient, followed at a later date by the application of a *test* and a *control* graft on each forearm.

The results of this type of experiment are summarized in Table II.

In the first instance, only one graft was used to sensitize the leukocyte donor. DNA-ase-treated extract was prepared from leukocytes obtained at the height of rejection of this graft, and injected into the shoulder of each of two nonsensitive recipients. Thirteen days after transfer, two test grafts and two control grafts were applied to the forearms of each recipient. It may be seen that, in this experiment, there is little difference between the rejection period of the test grafts (11 and 12 days, respectively) and that of the control grafts (nine days). *The results of this attempt at transfer of homograft sensitivity are interpreted as negative.*

In the second instance, an initial and a second set homograft from the same subject were applied to sensitize the leukocyte donor. The DNA-ase-treated extract was prepared by pooling leukocytes isolated at the height of the rejection periods of the initial graft and of the second set graft. The pooled leukocyte extract was divided into equal aliquots and injected into the skin of the shoulders of each of two recipients. Five days after transfer, two test and two control grafts were applied to the forearms of each recipient. Here again, there was little difference between the rejection period of the test grafts (11 and 10 days, respectively) and that of the control grafts (eight and seven days, respectively). *The results of this attempt at transfer of homograft sensitivity are interpreted as negative.*

2. Local technique of transfer. Leukocyte extracts were injected concentrically around test and control grafts.

In this series of observations, advantage is taken of the technique of Stetson and Demopoulos (15, 16) (in animals) of transfer of homograft sensitivity with serum. The present application is based on earlier findings in the transfer of tuberculin type allergy (22, 23) whereby the juxtaposition of antigen and transfer factor increases the sensitivity of the test system so as to allow a 10-fold reduction in the dosage of leukocytes ordinarily required to effect transfer by the systemic technique. In the local technique, the materials

(DNA-ase-treated leukocyte extracts, serum) to be tested for their capacity to transfer homograft sensitivity are injected around but not into test and control grafts, in halo fashion. The donor of the sensitive leukocyte extracts was sensitized with a first and a second set graft, as above, and the leukocytes were collected at the height of each rejection period, and pooled. Serum was obtained from the same donor at each of these bleedings, and was also pooled. The control donor of nonsensitive leukocyte extracts received no treatment.

Each of six nonsensitive individuals received a test and a control graft on each forearm. Three days later, when flow was established in each of the grafts, a concentric circle was formed in the recipient's forearms, about 20 mm. from the edges of each test and control graft, by the injection of either sensitive leukocyte extract or sensitive serum from the same donor, or nonsensitive leukocyte extracts prepared from a control donor.

The results of this experiment are summarized in Table III.

It may be seen that injection of sensitive leukocyte extracts into the recipient's forearm around test and control grafts in each of four recipients resulted in the accelerated rejection of the test grafts only, at the fourth to sixth day after grafting, while the control grafts survived for 8 to 10 days before rejection. It is notable that these effects upon the test grafts occurred within 24 to

TABLE III

*Effects of local transfer from leukocyte donor sensitized with two grafts**

Leukocyte donor	Sensitization of leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage injected around each graft site	Recipient No.	Day of graft rejection: Test graft	Day of graft rejection: Control graft	Interpretation of results
					ml.				
D4	2 Grafts	Local (pooled WBC first and second set rejection)	3 Days after grafts applied	Sensitive WBC extract†	0.22	R5	4–5	8	Positive
				Sensitive serum†	1.0		10	8	
D5	2 Grafts	Local (pooled WBC first and second set rejection)	3 Days after grafts applied	Sensitive WBC extract†	0.2	R6	4	10	Positive
				Sensitive serum	1.0	R7	10		Control
				Nonsensitive WBC extract	0.2		10		
D6	2 Grafts	Local (pooled WBC first and second set rejection)	3 Days after grafts applied	Sensitive WBC extract†	0.17	R8	5–6	10	Positive
				Sensitive WBC extract†	0.17	R9	5–6	10	Positive
				Sensitive serum	1.0	R10	10		Control
				Nonsensitive WBC extract	0.20		10		

* Skin donor	Skin recipient WBC or serum donor	WBC or serum recipient	Test graft donor	Control graft donor
Mat	Fol (D4)	Jaf (R5)	Mat	Rob
Abr	Jos (D5)	Pau (R6)	Abr	McK
		Tay (R7)	Abr	
			Abr	
Abr	Bar (D6)	McK (R8)	Abr	Pau
		Pac (R9)	Abr	Nig
		Lit (R10)	Abr	
			Abr	

† Injected around test and control graft, respectively.

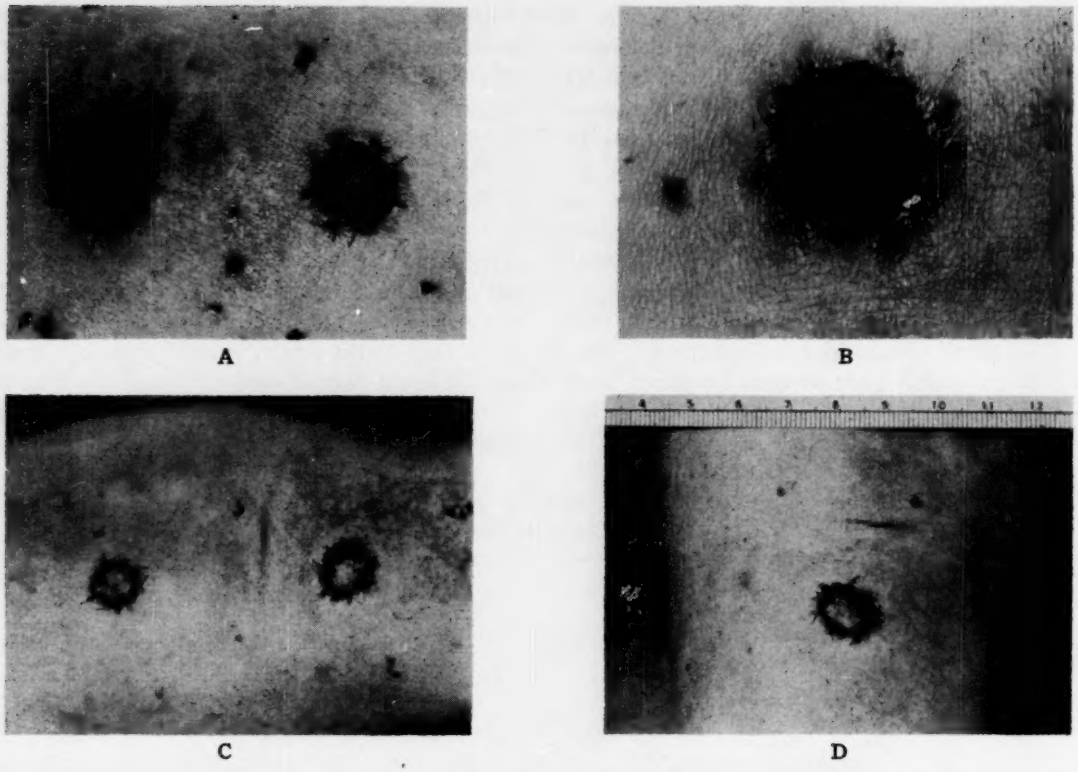

FIG. 1. LOCAL TRANSFER OF HOMOGRAFT HYPERSENSITIVITY WITH LEUKOCYTE EXTRACTS BUT NOT WITH SERUM FROM SENSITIZED DONOR [RECIPIENT NO. 5 (JAF)—TABLE III]

A. Left forearm—illustrating hemorrhagic appearance of test homograft (left) undergoing accelerated rejection and normal appearance of control homograft (right)—five days after application of both grafts and 48 hours after infiltration of sensitive leukocyte extract around both grafts. **B**. Close-up view of fully rejected test homograft in **A** above, six days after application of graft and 72 hours after transfer. **C**. Right forearm—illustrating normal appearance of test homograft (left) and control homograft (right) three days after application of grafts and immediately after infiltration of sensitive serum around both grafts. **D**. Close-up view of normal appearance of the test homograft in **C** above, four days after application of graft and 24 hours after serum transfer.

48 hours following the injection of sensitive leukocyte extracts.

The injection of sensitive serum or of nonsensitive leukocyte extracts around test grafts in this fashion had no effect upon their survival time (which was identical to that of the control grafts, *i.e.*, 10 days).

An example of this type of local transfer of homograft sensitivity is illustrated in photographs of the test and control grafts in Recipient No. 5 (R5) of Table III (see Figure 1). *The results of this attempt at local transfer of homograft sensitivity are interpreted as positive.*

B. Use of four consecutive skin homografts to sensitize the leukocyte donor. In this series of experiments, the donor of leukocytes was sensitized by a series of skin grafts from the same individual, applied on four successive occasions; each graft after the first underwent an accelerated rejection reaction. At the height of the fourth set rejection reaction, leukocytes were obtained, DNA-ase-treated leukocyte extracts were prepared, and injected into the nonsensitive recipient. In the experiments to follow, the leukocyte extract was injected into the shoulder of the recipient either 8 or 11 days before, or three days after application of test and control homografts to the respective recipients' forearms.

1. Relationship between the homograft rejection period and the capacity of leukocyte extracts to

TABLE IV

*Relationship between homograft rejection period and the capacity of leukocyte extracts to transfer hypersensitivity**

Leukocyte donor	Time of WBC collection	Method of transfer	Material used for transfer	Dosage WBC	Recipient No.	Day of homograft rejection: Test graft	Day of homograft rejection: Control graft	Interpretation
				ml.				
D7	At height of rejection of fourth graft	Systemic: 8 days before grafts	WBC extract	0.52	R11	4 4	11 11	Positive
		Systemic: 3 days after grafts	WBC extract	0.52	R12	4–5	8	Positive
	11 Days after above bleeding	Systemic: 11 days before grafts	WBC extract	0.50	R13	8	8	Negative
				0.50	R14	9	9	Negative
		Systemic: 3 days after grafts	WBC extract	0.50	R15	10 11	14 14	Negative
				0.50	R16	8 7	15 15	Questionable positive

* Skin donor	Skin recipient WBC donor	WBC recipient	Test graft donor	Control graft donor
Cox	Wal	McC (R11)	Cox	Nam
		Bro (R12)	Cox	Nam
		Fen (R13)	Cox	Nam
		Kel (R14)	Cox	Nam
		Gor (R15)	Cox	Nam
		Mel (R16)	Cox	Nam

transfer homograft hypersensitivity. The DNA-ase-treated leukocyte extracts used in this experiment were obtained from the same sensitized donor at two distinct periods: *1*) at the height of the fourth set accelerated homograft rejection period and *2*) 11 days after that period. Extract secured at each period was injected into the shoulder of a nonsensitive recipient, either 8 or 11 days before, or three days after the application of a test and control graft. The results are summarized in Table IV.

It may be seen that when leukocyte extracts obtained at the height of the accelerated rejection period of the fourth set graft were injected into the shoulder of a nonsensitive recipient eight days before the application of test and control grafts, this resulted in the selective accelerated rejection of the two test grafts at four days, while the two control grafts were unaffected and survived for 11 days. Extract of leukocytes obtained at the same bleeding were injected into the shoulder of a nonsensitive recipient three days after test and control grafts had been applied (at a time when blood flow was established in each graft). Within 24 hours after transfer the test graft underwent accelerated rejection (at the fourth day post-grafting) while the control graft survived for eight days.

The leukocyte extracts subsequently obtained from the same donor (D7) 11 days after the height of his fourth set rejection period were incapable of causing the accelerated rejection of test grafts in each of four additional recipients. This incapacity was evident whether the leukocyte extracts were injected 11 days before or three days after the application of test and control grafts.

With reference to Recipient No. 16 (R16), it should be mentioned that survival times of eight and seven days for test grafts as against 15 days for control grafts suggest a positive result. If it is indeed a positive result, it is surely a weak and questionable one, and further bears out the negative results achieved with the same leukocyte extracts, as an indication of the rapid decay in the capacity of Donor No. 7 (D7) leukocytes to transfer systemic sensitivity. It has not been given equal weight with other positive results, since the

response of the recipient to active sensitization by the graft itself may begin as early as the seventh day. It is for this reason that we have restricted the critical period for acceptance of a positive effect of transferred sensitivity to four to six days.

It is suggested from this experiment that an optimal time for securing leukocytes from sensitized donors capable of transferring sensitivity is at the height of the rejection period of the fourth set graft. These results also suggest a transient endowment of the leukocytes with the capacity to transfer sensitivity, when the donor is sensitized in this fashion.

2. Failure to transfer homograft sensitivity when the leukocyte donor is sensitized with three successive "white grafts." Since the schedule of sensitization employed above (*i.e.*, in which two to three weeks are allowed to pass between rejection of the last and application of the next successive graft) requires several months, an effort was made to sensitize leukocyte donors over a shorter period. To accomplish this, the interval between rejection of the preceding graft and application of the next successive graft was shortened to seven days.

It was observed, however, that when second, third and fourth grafts were applied in this fashion, the sequence of initial vascularization (three days) followed by accelerated rejection (four to six days) did not occur. Instead, each graft after the first was not vascularized, and remained white and opaque from the time of application. This has been termed the "white graft" reaction, and its occurrence and possible significance have been described elsewhere (40, 41).

Leukocytes and erythrocytes in plasma were obtained from a donor sensitized in this fashion, at the height of the fourth "white graft" reaction. The DNA-ase-treated leukocyte extracts were injected into the shoulder of four nonsensitive recipients and an equivalent amount of erythrocytes and plasma was similarly injected into an additional nonsensitive recipient. Each recipient had received a test and control graft on each forearm four days before the attempts at transfer were made. The results of this experiment are summarized in Table V.

TABLE V

Effects of systemic transfer from leukocyte donor sensitized with "white grafts" *

Leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage WBC	Recipient No.	Day of homograft rejection: Test graft	Day of homograft rejection: Control graft	Interpretation of results
				ml.				
D8	Systemic	4 Days after grafts applied	Sensitive WBC extract	0.6	R17	7	9	Negative
			Sensitive WBC extract	0.59	R18	7	6	Negative
			Sensitive WBC extract	0.63	R19	6†	6†	Negative
			Sensitive plasma and RBC	2.5 0.6	R20	9	8	Control
			Sensitive WBC extract	0.59	R21	9	8	Negative

* Skin donor	Skin recipient WBC or control plasma donor	WBC or plasma recipient	Test graft donor	Control graft donor
Car	Cas	Els	Car	Mat
		New	Car	Mat
		Ren	Car	Mat
		Gou	Car	Mat
		O'Br	Car	Mat

† Technically unsatisfactory graft application.

It may be seen that neither the putatively sensitive leukocyte extract nor the erythrocytes and the plasma obtained from the same donor had any effect on the survival of test as compared to control grafts.

It is concluded that the schedule of sensitization by means of skin grafts cannot be hurried. The results suggest that the accelerated rejection reaction is superior to the "white graft" reaction as a means of sensitizing the leukocyte donor.

3. Corroboration of the efficacy of four successive grafts and accelerated rejection as a means of sensitizing the leukocyte donor. Following the results described in Section *2* above, the next donor of leukocytes was sensitized exactly as described under Section *1* above (*i.e.*, by a series of four successive grafts, the latter three being applied at intervals of two to three weeks after rejection of the preceding graft, and each undergoing accelerated rejection). The DNA-ase-treated extract was prepared from leukocytes obtained at the height of accelerated rejection of the fourth set graft. The leukocyte extract was injected into the shoulders of four nonsensitive recipients. Eight days following transfer, a test and control graft were applied to both forearms of each recipient. The results of this experiment are summarized in Table VI.

It may be seen that in each recipient, the test graft was rejected in an accelerated fashion 24 hours after transfer (and four days postgrafting) while the control grafts did not undergo accelerated rejection. It should be noted that technical difficulties at the time of grafting contributed to the rejection of the control graft in Recipient No. 25 (R25) at seven days.

From these results, it is concluded that homograft sensitivity is amenable to transfer with leukocyte extracts when the latter are obtained from an adequately sensitized donor. Examples of this type of transfer are illustrated in Figure 2.

C. Summary of results of transfer experiments. The results of attempts at transfer of homograft sensitivity are listed in Table VII.

It may be seen that neither one nor two successive grafts are sufficient to endow the leukocyte extracts of donors sensitized in this fashion with the capacity to transfer homograft sensitivity *systemically*. However, two successive grafts are sufficient to sensitize donor leukocyte extract to such an extent as to allow a *local* transfer of sensitivity. Serum obtained from the same sensitive donors, as well as leukocyte extracts obtained from nonsensitive donors, are incapable of transferring homograft sensitivity, even when favored by the highly sensitive technique of local transfer.

It is also noted that systemic homograft sensitivity can be transferred by leukocyte extracts if a dosage of four successive grafts is used to sensitize the leukocyte donor, and each graft after the first has undergone accelerated rejection.

The effects of transfer of local or of systemic sensitivity to recipients bearing vascularized grafts appear promptly within 24 to 48 hours following transfer. When graft application follows the transfer of sensitivity, the test graft is seen to be vascularized by the third day and to undergo accelerated rejection within 24 hours after this event.

From one group of our observations, it would appear that the capacity of the sensitized donor's leukocytes to transfer sensitivity is short-lived. From another group of observations, it would also appear that graft rejection via the "white graft" reaction does not serve as an efficient stimulus for sensitizing the leukocyte donor to the extent necessary to effect a successful transfer of sensitivity with leukocyte extracts.

DISCUSSION

The rapid onset of accelerated rejection of test grafts 24 to 48 hours after transfer, in subjects bearing fully vascularized test and control grafts, may be taken as good evidence of a specific passive transfer of sensitivity. The prompt effects achieved by this method of transfer exclude the participation of antigen in the results observed. However, the accelerated rejection of test grafts placed upon recipients eight days after transfer implies a more intimate participation of the recipient in the inauguration of the mechanism of rejection. This is suggested by the fact that in each of such instances, the test graft became vascularized, and flow between host and graft was established usually 24 hours before the onset of accelerated rejection. Inherent in the design of the latter type of transfer experiment is the remote possibility that antigen, however minute in quantity, may be carried over in the sensitized leukocyte extract used to transfer sensitivity. If

TABLE VI

*Effects of systemic transfer from leukocyte donor sensitized with four grafts via accelerated rejection reaction**

Leukocyte donor	Type of transfer	Time of transfer	Material used for transfer	Dosage WBC	Recipient No.	Day of homograft rejection: Test graft	Day of homograft rejection: Control graft	Interpretation of result
				ml.				
D9	Systemic	8 Days before grafts applied	WBC extract	0.7	R22	4	11	Positive
				0.7	R23	4	8	Positive
D10	Systemic	8 Days before grafts applied	WBC extract	0.9	R24	4	13	Positive
				0.9	R25	4	7†	Positive

* Skin donor	Skin recipient WBC donor	WBC recipient	Test graft donor	Control graft donor
Fol	Jos (D9)	Car (R22)	Fol	Rob
		Wes (R23)	Fol	Rob
Jos	Fol (D10)	She (R24)	Jos	Rob
		Rub (R25)	Jos	Rob

† Partial separation from bed on third day due to unsatisfactory technique.

this possibility is proved, then the possibility of active sensitization in the recipient of this method of transfer will have to be excluded. Experiments are planned to assess the capacity of DNA-ase-treated leukocyte extracts in this regard.

The requirement of four successive grafts to

TABLE VII

Summary of results of transfer of specific sensitivity to skin homografts

Mode of sensitization and time of collection of WBC from donor	Method of transfer	Material used for transfer	Results of transfer: No. of recipients	Results of transfer: No. of positive transfers
First set rejection	Systemic	Sensitive WBC extract	2	0
Second set rejection	Systemic	Sensitive WBC extract	2	0
First and second set rejection	Local	Sensitive WBC extract	4	4
		Nonsensitive WBC extract	2	0
		Sensitive WBC extract on control graft	4	0
		Sensitive serum on test graft	3	0
Fourth set rejection	Systemic	Sensitive WBC extract	6	6
11 Days after fourth set rejection	Systemic	Sensitive WBC extract	4	0
5 Days after fourth set "white graft" application	Systemic	Sensitive WBC extract	4	0
		Sensitive plasma and RBC	1	0

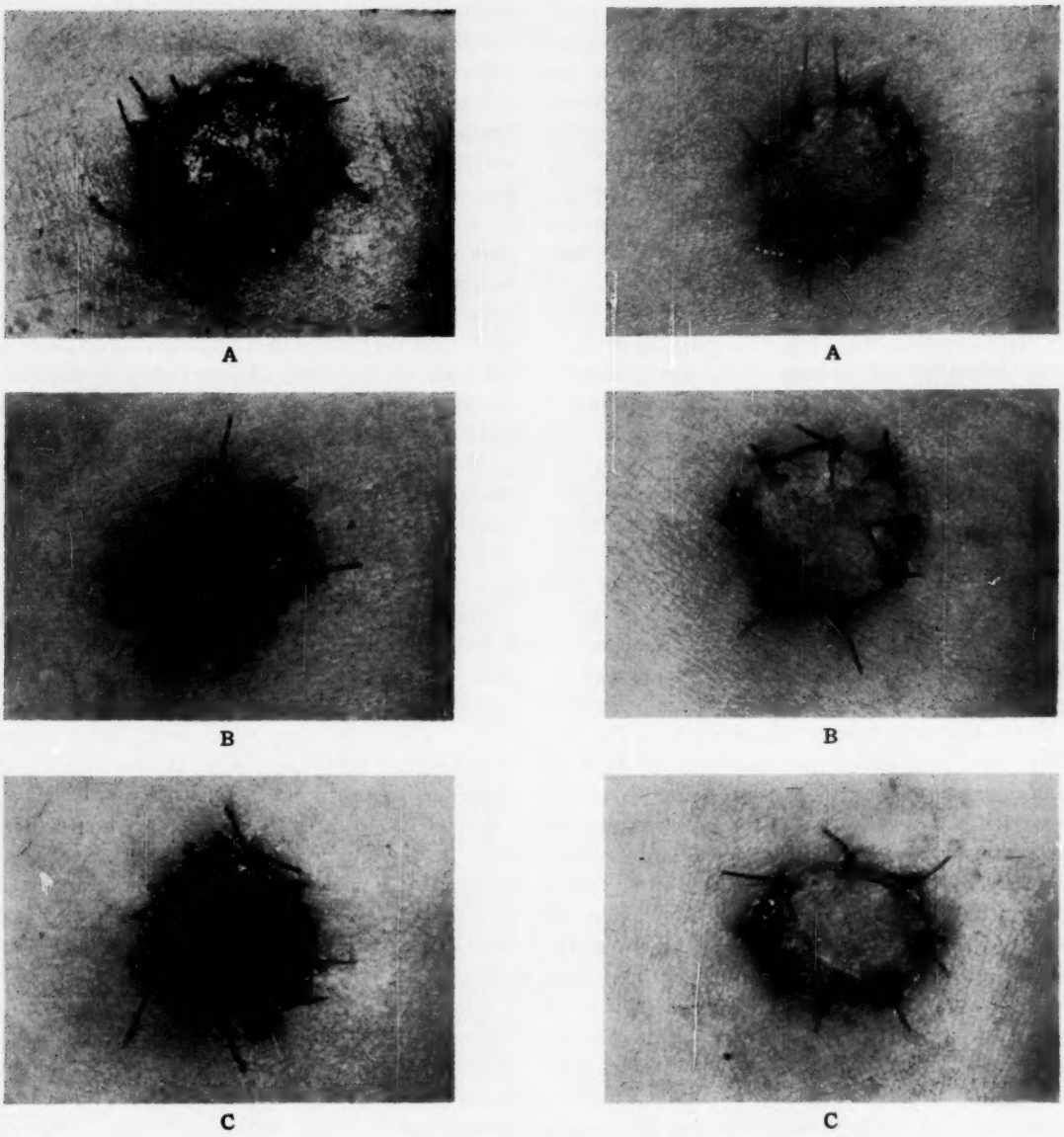

FIG. 2. SYSTEMIC TRANSFER OF HOMOGRAFT HYPERSENSITIVITY WITH LEUKOCYTE EXTRACTS OBTAINED FROM SENSITIZED DONOR—TABLE VI

A. Recipient No. 22 (Car), **B**. Recipient No. 23 (Wes) and **C**. Recipient No. 24 (She)—illustrating in each recipient the hemorrhagic appearance of the test homograft (left) undergoing accelerated rejection and normal appearance of the control homograft (right)—four days after application of each graft and 12 days after transfer to each recipient, respectively.

sensitize the donor's leukocytes to the extent that a systemic transfer of sensitivity is possible suggests that the application of orthotopic skin homografts is not the most efficient means of sensitization for this purpose. Another indication of the critical importance of quantitative variables in governing the success of attempts at transfer may be gained from the observations made employing the local technique of transfer. In this instance, only two successive grafts were sufficient

to endow leukocytes with the capacity to transfer *local* but not *systemic* sensitivity. It may be that, for convenience and ease of interpretataion of results, this method of transfer is to be preferred over the systemic transfer of homograft sensitivity. However, in a preliminary study of this kind, there are good theoretical reasons for also having successfully effected the systemic transfers.

One of the many great advantages of using animal species for a more precise demonstration of the transfer of homograft sensitivity has been the use of inbred strains. This is not possible in man; here, the tempo and intensity of the host's immune response to homografts, and the capacity to transfer such sensitivity, will parallel the degree of genetic disparity between the individuals studied. The greater the genetic differences (*i.e.*, the less the number of tissue transplantation antigens shared) between the individuals studied, the more abrupt and intense will be the reaction of one to a graft from the other. This consideration, coupled with the fact that the experimental demonstration of the individual specificity of homograft responses has not been as extensively studied in man (39–41, 43) as in animal species (4, 6, 7, 10, 11), introduces inescapable variables for the type of experimental observation reported in this study. Nevertheless, since the effects of transfer on the test grafts have been judged as significant only in terms of their relation to the behavior of control grafts on the same recipient, it is felt that the bearing of such variables on the observations reported, although not eliminated entirely, has been greatly diminished.

The observations reported here are in general agreement with Mitchison and Dube's (18, 19) findings on the transfer of tumor homograft sensitivity in mice, Billingham, Brent and Medawar's (10, 11) findings on the transfer of skin homograft sensitivity in mice and Brent, Brown and Medawar's (12) findings on the transfer of skin homograft sensitivity in the guinea pig. The effectiveness of peripheral blood leukocytes in human subjects is in contrast to the requisite for successful transfers in animal species that the leukocytes be obtained from lymph nodes draining the graft (10, 18, 19), or from the spleen (10) of the sensitive donor. The present demonstration of the critical role of timing in determining effective sensitization, transfer, and test of transferred sensitivity, suggests an approach to the resolution of this species difference.

The loss of capacity to transfer sensitivity in Donor No. 7 (D7), Table IV, suggests that sensitization and desensitization to skin homografts may proceed *pari passu*, and that the success or failure of transfer may depend upon which state is in the ascendancy at the time of leukocyte collection. A similar loss in the capacity of leukocytes to transfer delayed tuberculin hypersensitivity has been produced experimentally *in vitro* (38) and *in vivo* (44) in man following exposure to tuberculin and resultant desensitization of the leukocytes.

The property of DNA-ase-treated leukocyte extracts to transfer homograft sensitivity in man is a striking departure from the experience in animal species, where intact, viable cells are necessary for this effect (9–11). Such a species difference may be a reflection of yet another parameter of the greater relative ease with which man can become sensitized to a wide variety of foreign materials (bacteria, simple chemicals) (45). This is believed the more likely in respect to homograft sensitivity, since it has been shown earlier to be operative in the transfer of delayed bacterial (30, 31) and fungal (26) hypersensitivities. In the latter systems of delayed allergy, the efficacy of leukocyte extracts in the transfer of sensitivity has been repeatedly demonstrated (26, 30, 31, 38) and more recently confirmed elsewhere (32–34), in human subjects.

There has been no attempt to measure antibodies in the serum of recipients of transfer in this study, in view of the negative results of attempts at transfer with serum, and the failure to demonstrate conventional serum antibody following transfer of bacterial allergy in humans with leukocyte extracts (31) or viable cells (17, 25) when the leukocytes have been obtained from the *peripheral* blood.

The failure to transfer homograft sensitivity with extract obtained from "white graft" reactors suggests that this type of homograft response may not be mediated by "cell-bound" antibody.

It has been the purpose of this study to adapt the highly sensitive transfer system in human subjects to an evaluation of the possible mechanisms

of homograft rejection—an adaptation which broadens the biological properties of transfer factor and allows the use of extracts of leukocytes. The latter device gives promise of sorting out the antibody(ies) that mediates homograft rejection, the cells concerned in the process, and perhaps the antigen(s) of tissues concerned with the induction of this immune response. In any event, insofar as homograft sensitivity is amenable to transfer with extracts of leukocytes, an approach has been provided whereby the progress made with this system in humans in relation to delayed bacterial hypersensitivity can be applied directly to the study of the immunology and pathogenesis of the homograft rejection phenomenon, as a general biological prototype of altered tissue reactivity.

SUMMARY AND CONCLUSIONS

1. Skin homograft sensitivity (accelerated homograft rejection) has been transferred to nonsensitive recipients by means of an injection of desoxyribonuclease-treated leukocyte extracts prepared from sensitive donors: *a*) in four out of four consecutive attempts by employing a local technique of transfer and a dosage of two grafts to sensitize the leukocyte donor; *b*) in six out of six attempts by employing the systemic technique of transfer and a dosage of four grafts to sensitize the leukocyte donor.

2. A dosage of *one* or *two* grafts is not sufficient to sensitize the leukocyte donor so as to allow a systemic transfer of sensitivity.

3. A dosage of four successive grafts is sufficient to sensitize donor leukocytes so as to permit systemic transfer of sensitivity *only* if the repeat set grafts undergo accelerated rejection, and *not* if "white graft" reactions are produced.

4. Serum from sensitized donors or leukocytes from nonsensitive donors are incapable of transferring homograft sensitivity.

5. The results of transfer of homograft sensitivity by means of leukocyte extracts generally parallel those obtained in man following similar transfers of bacterial (tuberculin, streptococcal proteins, diphtheria toxoid) and fungal (coccidioidin) hypersensitivities of the delayed type. This finding fulfills a critical criterion relating delayed hypersensitivity of the tuberculin type to homograft sensitivity.

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SEQUENTIAL MECHANISMS IN THE ENHANCED ABSORPTION OF VITAMIN B_{12} BY INTRINSIC FACTOR IN THE RAT *

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Pernicious anemia, a clinical state of vitamin B_{12} deficiency, is characterized by a virtually complete absence of gastric intrinsic factor secretion, resulting in marked inability of the patient to absorb the low concentrations of vitamin B_{12} present in usual dietary sources. Intrinsic factor deficiency is also produced by total gastrectomy in the rat and, as in the patient, can be temporarily abolished by the oral administration of intrinsic factor. A number of facts concerning the nature and function of intrinsic factor have been discovered by observations on patients (1–2) and, recently, on gastrectomized rats (3–5).

Intrinsic factor preparations of various species —man, hog, rat and dog—are capable of binding vitamin B_{12} (6–9) as are other substances such as food proteins which, however, lack intrinsic factor activity (10, 11). Vitamin B_{12} bound by intrinsic factor is preferentially absorbed over unbound vitamin when both are simultaneously administered in pernicious anemia (12) as well as in the intestine of the gastrectomized rat (13). These facts suggest that normally the first task of the gastric intrinsic factor may be to compete for the vitamin B_{12} of ingested animal protein with which the vitamin is associated or bound. For convenience in reporting the present experiments, we shall refer to this competition as Phase I of the hypothetical action of intrinsic factor. That vitamin B_{12} bound to intrinsic factor is preferentially absorbed also suggests that the vitamin must approach, adhere to, and perhaps even penetrate the intestinal wall in combination with intrinsic factor. It has been stated that binding of vitamin B_{12} to rat intestinal segments *in vitro* is greatly enhanced by both hog (14) and rat (15) intrinsic factor-containing preparations. This process apparently requires the presence of calcium ions (14). However, because hog intrinsic factor does not enhance absorption of vitamin B_{12} by the gastrectomized rat (3–5), the physiological significance of experiments with hog stomach preparations requires evaluation. Thus adherence of vitamin B_{12} to the intestinal wall after binding to intrinsic factor may be only a preparatory step (Phase II) for subsequent species-related transport across the intestinal wall. At any rate, there is evidence from observations in both man (16, 17) and in the rat (18) that a process concerned with the absorption of vitamin B_{12} takes place in the intestinal wall and requires up to four hours. Species-related activity would be expected to be characteristic of a process occurring within a tissue but not necessarily upon its surface, in this case the small intestine. Consequently, as in a recent preliminary report of this work (19), we shall refer for convenience to the transport of the vitamin across the intestine and into the blood stream as Phase III of the action of intrinsic factor.

MATERIALS AND GENERAL METHODS

Various sources of intrinsic factor. Rat gastric juice was collected by the method of Shea (20) using ether anesthesia. Five hours of such pyloric ligation was found to give a 5 to 10 ml. yield from the distended stomach of a fasted but well hydrated rat. Using 20 per cent sodium hydroxide solution, the juice was immediately neutralized to pH 7.0, as determined on a Beckman pH meter and kept frozen in aliquots at $-20°$ C. until used.

Rat stomach extract was prepared from freshly frozen rat stomachs stored at $-20°$ C. The stomachs were thawed, homogenized in water in a Waring blendor, centrifuged, and the sediment discarded. One part of concentrated hydrochloric acid solution was added to each nine parts of the extract, and the precipitate was removed by centrifugation. The extract was then neutralized to pH 7.0 with 20 per cent sodium hydroxide (w/v), dialyzed against water at 4° C. for 18 hours, and thereafter concentrated at room temperature in a cellophane casing suspended before an electric fan to a volume correspond-

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ing to 2 ml. of extract per stomach. One-half ml. of this material was found to be active in reducing the 100 per cent fecal excretion of radioactivity of gastrectomized rats fed 0.015 μg. of radioactive vitamin B_{12} to 70 per cent of the administered dose. Normal rats excrete about 50 per cent of this dose of Co^{60}–B_{12}.

Human gastric juice was collected by Histalog® [1] stimulation of normal donors after gastric intubation. The juice was filtered through cheesecloth, allowed to stand at pH 10 for 20 minutes in order to destroy pepsin (21) and then neutralized to pH 7. The gastric juice from five donors was pooled and stored frozen at −20° C. in small aliquots.

Hog intrinsic factor preparation No. Wes 671-A is a whitish powder,[2] reported to have no inhibitory action in pernicious anemia. This preparation was suspended in a concentration of 0.1 per cent (w/v) in water kept at 4° C. and diluted 1:20 in water just before use. Four mg., when given with 15 μg. of vitamin B_{12} is stated to represent 1 U.S.P. unit (oral) of Vitamin B_{12} with Intrinsic Factor Concentrate.

Other experimental reagents. Rat intestinal extract was prepared by homogenizing the entire small intestine of a 250 Gm. male rat in 50 ml. of distilled water in a Waring blendor. This homogenate was then centrifuged in a laboratory angle centrifuge, and the slightly opalescent supernatant was frozen at −20° C. in small aliquots.

Krebs-Ringer phosphate at pH 7.4 was prepared fresh daily, from stock solutions, as described by Umbreit, Burris and Stauffer (22).

Other assay methods employed. Radioactive vitamin B_{12}, Co^{60}–B_{12}, with a specific activity of approximately 1 μc. per μg,[3] was used in a concentration of 0.03 μg. per ml. of water. Partition coefficients between benzyl alcohol and phosphate buffer at pH 7.0 ranged from 0.75 to 0.80, indicating that the solution contained between 90 and 100 per cent cyanocobalamin (23). Radioactive counting of various solutions containing Co^{60}–B_{12} was carried out in a well-type scintillation counter.

Nonradioactive vitamin B_{12} was determined microbiologically using *Euglena gracilis* var. bacillaris according to the method of Ross (24).

INVESTIGATIONS

The present experiments represent attempts to add further information concerning the characteristics of each of the three hypothetical phases of intrinsic factor action mentioned in the introduction.

[1] Parenteral preparation of 3-beta-aminoethylpyrazole dihydrochloride (Lilly).

[2] Obtained through the kindness of Dr. Thomas Jukes, American Cyanamid Company, Pearl River, N. Y.

[3] Obtained from Merck and Company, Rahway, N. J.

Phase I: Competition of intrinsic factor for vitamin B_{12} *of ingested food proteins*

In these experiments the rates of diffusion through cellophane membranes of vitamin B_{12} from homogenized suspensions of lean beef and pork muscle and from a solution of casein were compared with the diffusion rate of vitamin B_{12} from normal human gastric juice at various pH values.

Experiments with added Co^{60}–B_{12}. One per cent Hammarsten casein solution, 1 per cent lean beef muscle, and the centrifuged supernatant as well as the sediment of a 1 per cent lean pork muscle suspension, respectively, were incubated with 0.006 μg. of Co^{60}–B_{12} at room temperature in the dark for one hour. Equal volumes of these solutions were then placed in screw-capped test tubes, and 0.5 ml. of neutralized human gastric juice was added to selected tubes. An equal volume of distilled water was added to the remainder of the tubes. Visking casing, 8 mm. in diameter and 15 cm. in length with the ends tightly tied in order to form a bag, was used as a semipermeable membrane. In order to equalize osmotic pressure differences, equal volumes of appropriate aliquots of the casein, pork or beef muscle suspensions, but without added Co^{60}–B_{12}, were placed inside the visking bags. The bags were folded over, with an air bubble in each limb, and inserted into the test tubes, which were then closed with "parafilm" and a screw cap. Dialysis was then carried out while the test tubes, which contained an air space, were rotated end over end at the rate of 6 rpm in an incubator in the dark at 37° C. for five hours.

Duplicate experiments were run at pH 3.5 and pH 7.0, by adjustment of the original suspension before division between the test tube and the interior of the cellophane bag with 2 N hydrochloric acid or with 0.1 N sodium hydroxide solution. In other experiments preliminary incubation of cellophane bags in 0.85 per cent saline at pH 3.5 for five hours demonstrated no detectable effect of this treatment on the membrane during subsequent dialyses at pH 3.5 or pH 7.0.

At the conclusion of each experiment the cellophane bag was removed from each test tube, quickly rinsed under running water and wiped dry. Two ml. of its contents, as well as of its

TABLE I

Radioactivity transferred during dialysis from "outside solution" (containing added Co^{60}–B_{12}) to an equal volume of "inside solution" (containing no added Co^{60}–B_{12})

Composition of outside solution	pH	Composition of inside solution	Increase in radioactivity of inside solution
			%
Water	7.0	Water	47.5
Casein	7.0	Water	22.0
Casein	3.5	Water	32.0
Casein	7.0	Water + NHGJ*	25.0
Casein	3.5	Water + NHGJ	42.0
Casein + NHGJ	7.0	Casein	2.6
Casein + NHGJ	3.5	Casein	2.6
Pork muscle supernatant	7.0	Pork muscle supernatant	38.8
Pork muscle supernatant	3.5	Pork muscle supernatant	42.8
Pork muscle supernatant	7.0	Pork muscle supernatant + NHGJ	72.0
Pork muscle supernatant	3.5	Pork muscle supernatant + NHGJ	81.5
Pork muscle sediment	7.0	Pork muscle sediment	4.1
Pork muscle sediment	3.5	Pork muscle sediment	36.0
Pork muscle sediment	7.0	Pork muscle sediment + NHGJ	5.5
Pork muscle sediment	3.5	Pork muscle sediment + NHGJ	75.5
Beef muscle suspension	7.0	Beef muscle suspension	35.0
Beef muscle suspension	3.5	Beef muscle suspension	39.5
Beef muscle suspension	7.0	Beef muscle suspension + NHGJ	57.0
Beef muscle suspension	3.5	Beef muscle suspension + NHGJ	78.0

* Normal human gastric juice.

corresponding test tube, were then measured out using volumetric pipettes. The radioactivity of each solution was determined. The exchange of radioactivity from outer to inner solution was recorded as a percentage of the initial radioactivity of the outer solution. The sums of the radioactivity in both the inner and the outer solutions represented approximately all of the radioactivity originally introduced.

In certain experiments, dialysis was carried out in the dark without rotation at 37° C. for seven hours and at 4° C. for 18 hours. These procedures were found to give results identical to those of the procedure described above.

Results. As shown in Table I, added Co^{60}–B_{12} diffused slowly at pH 7.0 from casein and pork muscle, and with less difficulty from beef muscle and from the soluble substances in pork muscle. This diffusibility was greater when dialysis took place at pH 3.5 and was also increased at both pH 3.5 and 7.0 by the presence of human gastric juice devoid of peptic activity on the opposite side of the cellophane membrane. On the other hand, when the same amount of Co^{60}–B_{12} was added to human gastric juice there was essentially no diffusion of the vitamin into casein at either pH 3.5 or pH 7.0.

Experiments with native vitamin B_{12}. As a check on the studies with added Co^{60}–B_{12}, similar dialysis experiments were carried out using suspensions of beef and pork muscle in which the native vitamin B_{12} exchanged was determined microbiologically. Suspensions of 2 per cent beef and pork muscle were placed in test tubes and dialyzed against an equal volume of 12.5 per cent human gastric juice of known vitamin B_{12} content at pH 6.0 and at pH 1.5. Twenty-five per cent normal human gastric juice in water was dialyzed against water at pH 6.0 and 1.5, respectively. After dialysis, the solutions inside the cellophane bags were heated to 100° C. in order to free the vitamin and were then analyzed microbiologically for their gain in vitamin B_{12} content.

Results. As shown in Table II, dialysis of normal human gastric juice against beef and pork muscle suspensions at both pH 6.0 and 1.5 resulted in a marked increase in the native vitamin B_{12} content of the gastric juice. This loss of vitamin B_{12} to gastric juice appeared to be en-

TABLE II

Native vitamin B_{12} transferred during dialysis from "outside solution" to an equal volume of "inside solution"

Composition of outside solution	pH	Inside solution: Composition	Inside solution: Vitamin B_{12} Before dialysis	Inside solution: Vitamin B_{12} After dialysis
			μμg./ml.	μμg./ml.
Pork muscle suspension, 25% in water	6.0	NHGJ*	104	1,304
	1.5	12.5% in water	104	1,384
Beef muscle suspension, 25% in water	6.0	NHGJ	104	1,904
	1.5	12.5% in water	104	7,144
Water	6.0	NHGJ	208	208
	1.5	25% in water	208	208

* Normal human gastric juice.

hanced at low pH values as in the experiment with added Co^{60}–B_{12}. On the other hand, dialysis of gastric juice against water at pH 1.5 and 6.0 resulted in no detectable loss of its native vitamin B_{12} content at either pH.

Phase II: Enhanced adsorption by the intestinal wall of vitamin B_{12} bound to intrinsic factor

In these experiments intraluminal perfusions were carried out through cannulae inserted into paired loops of rat ileum with intact blood supply. The effect of sources of intrinsic factor and of other substances upon the uptake of Co^{60}–B_{12} by the intestine was determined by measurement of the differences in radioactivity of the paired intestinal segments removed at the end of each experiment.

The perfusion technique employed was that of Nieweg, Shen and Castle (13) except that it was conveniently discovered that intestinal segments of normal fasted rats, like those of gastrectomized rats, were sensitive to the influence of rat intrinsic factor. Consequently, intact male Sprague-Dawley rats weighing 150 to 250 Gm. were anesthetized with pentobarbital, 3.5 mg. per 100 Gm. of rat, given intraperitoneally. Two segments of small intestine, 10 cm. in length, were then isolated between fine plastic inflow and outflow catheters tied in place and carefully positioned to permit free passage of fluid from above downward through the lumen of each segment. By means of a pair of 1 ml. tuberculin syringes equipped with three-way stopcocks and rigidly supported by a burette stand, test and control solutions could be simultaneously perfused at similar rates controlled by a stop watch through one or the other of the pair of intestinal segments. Although no consistent differences were detected, the upper and lower intestinal loops were alternately selected in successive animals for perfusion of the test and control solutions, respectively.

At the beginning of an experiment the intestinal loops were rinsed by perfusion with 2 ml. of warm Krebs-Ringer phosphate solution at the rate of 0.5 ml. per minute. The animal was then left undisturbed for three minutes, following which the perfusion of the test fluid was begun in one, while the control fluid was perfused at an identical rate through the other isolated loop. At first, 0.25 ml. of each perfusion fluid was injected every 15 seconds for one minute. This was then followed by 0.20 ml. of the same fluid at one minute intervals for 15 minutes. Thus, in all, 4.0 ml. of each solution was perfused in 17 minutes through each loop of intestine. Usually the perfusion fluid and its control were constituted to approximate Krebs-Ringer phosphate solution. Invariably each contained 0.015 μg. of Co^{60}–B_{12}.

At the end of the perfusion the loops were "rinsed" by perfusion with 6 ml. of isotonic saline and then gently emptied of liquid by the introduction of 6 ml. of air through the inflow cannula. Each loop was then removed from the animal preparation, turned inside out on a metal rod, taken off, placed in a 25 ml. Erlenmeyer flask and shaken gently for five minutes in 4 ml. of the rinsing solution required by the particular experiment. The tissue was then removed,

drained of rinsing fluid, and dissolved in a test tube containing a weighed amount of concentrated sulfuric acid. The weight of each loop was determined by difference, and its radioactivity estimated in a scintillation counter.

Effects of rat and human gastric juice and of a hog intrinsic factor preparation on uptake of Co^{60}–B_{12}. In these experiments one intestinal loop was perfused with 4.0 ml. of Krebs-Ringer phosphate solution containing 2.0 ml. of rat gastric juice or other source of intrinsic factor while the other loop was perfused with a solution identical except that the source of intrinsic factor was replaced by an equal volume of water. In other experiments 0.5 ml. of human gastric juice or 1.0 ml. of a 1 : 20 dilution of the hog intrinsic factor preparation was substituted for the rat gastric juice.

Results. Inspection of Figure 1 shows that rat gastric juice clearly augmented the uptake of Co^{60}–B_{12} by the intestinal wall. Augmentation by human gastric juice was less impressive and, as shown in Figure 3, with twice the amount of gastric juice was even less. With the hog intrinsic factor preparation, enhancement was undetectable.

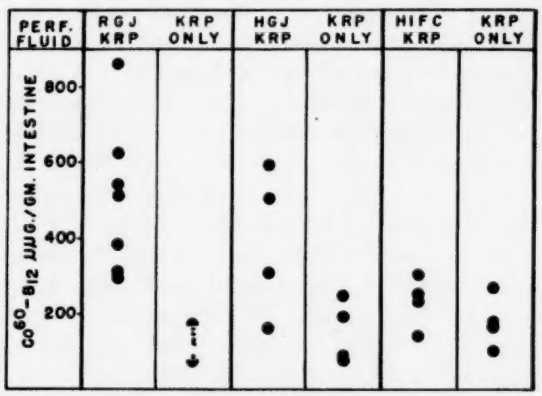

FIG. 1. EFFECT OF THREE DIFFERENT SPECIES OF INTRINSIC FACTOR GIVEN AS RAT GASTRIC JUICE (RGJ), HUMAN GASTRIC JUICE (HGJ) AND HOG INTRINSIC FACTOR CONCENTRATE (HIFC), RESPECTIVELY, UPON THE ADSORPTION OF Co^{60}–B_{12} BY ONE OF A PAIR OF LOOPS OF RAT INTESTINE

Each circle represents the amount of Co^{60}–B_{12} adsorbed during a single perfusion experiment with the species of intrinsic factor indicated (left-hand columns). The control value with Krebs-Ringer phosphate (KRP) solution alone in the other member of a given pair of loops is shown by one of the circles in the adjacent (right-hand) columns. Each perfusion fluid contained 0.015 µg. of Co^{60}–B_{12}. It appears that rat gastric juice is active, human gastric juice less so, and the hog intrinsic factor preparation not at all.

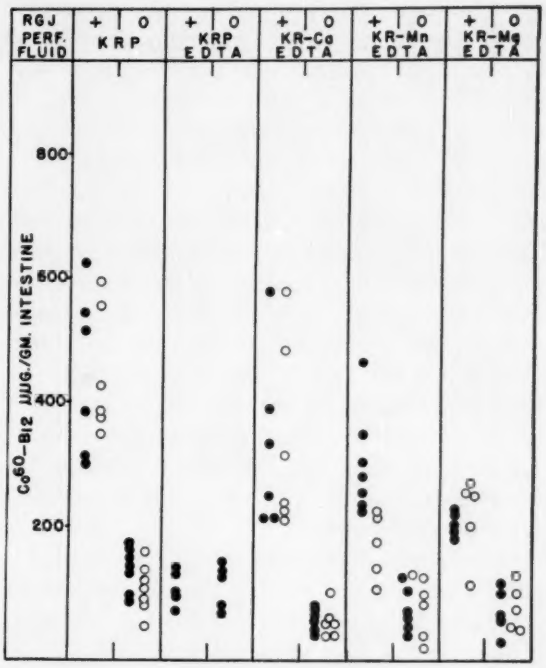

FIG. 2. EFFECT OF CALCIUM LACK AND OF SUBSTITUTION OF MANGANESE AND OF MAGNESIUM IONS FOR CALCIUM IONS UPON THE ADSORPTION OF Co^{60}–B_{12} WITH (LEFT-HAND COLUMNS) AND WITHOUT (RIGHT-HAND COLUMNS) RAT GASTRIC JUICE (RGJ)

Each circle represents the amount of Co^{60}–B_{12} adsorbed by half of each member of a pair of loops of rat intestine. After the perfusion, half of each loop was rinsed in Krebs-Ringer phosphate (KRP) solution (solid circles), the other half loop in disodium ethylenediaminetetraacetate (EDTA) solution (open circles). Thus, in each experiment the adsorbed radioactivity of each half of each pair of loops was measured; in all, four specimens. It appears that calcium is required for the adsorption of Co^{60}–B_{12} under the influence of rat gastric juice and that managanese and perhaps magnesium may partially substitute for calcium ions.

Effect of calcium and other bivalent cations on uptake of Co^{60}–B_{12}. Suggested by the work of Herbert (14, 25), studies were made concerning the effect of bivalent cations. Special Krebs-Ringer phosphate solutions were prepared in which disodium ethylenediaminetetraacetate (EDTA) replaced sodium chloride in the initial rinsing fluid, as well as in the perfusion fluid. In some

experiments the standard, and in others the special EDTA-containing, Krebs-Ringer phosphate solution was employed in both loops, while in one loop the perfusion solution contained 2.0 ml. of neutralized rat gastric juice and the other contained an equal added volume of water. After the perfusion each intestinal loop was removed from the animal and everted on a metal rod. Then, in all the experiments except those in which the perfusion fluid contained sodium EDTA, the loop was carefully divided in half longitudinally with a safety razor blade, while still on the rod. Each half-loop was then washed in Krebs-Ringer phosphate solution for five minutes, the other half in an aqueous solution containing 100 mMoles per L. of sodium EDTA at pH 7.0. Thereafter, the radioactive content of each loop or half-loop was determined as usual.

Other special Krebs-Ringer solutions were prepared in which the EDTA was balanced by an equivalent amount of calcium (as $CaCl_2$), manganese (as $MnSO_4$), or magnesium (as $MgSO_4$). Phosphate was omitted from these solutions in order to prevent precipitation, and they were adjusted to pH 7.5 with 2 N NaOH. The EDTA exerted sufficient buffering power to maintain the pH of the effluent from the intestinal loop between 7.0 and 7.5 as determined by nitrazine paper. Both intestinal loops were initially rinsed and perfused in different experiments with either the standard or a special Krebs-Ringer solution. After the completion of the perfusion, half of each loop was rinsed in the standard Krebs-Ringer phosphate solution and half in the sodium EDTA solution. Finally the radioactivity of each half-loop was determined in the usual fashion.

Results. As shown in Figure 2 augmentation of Co^{60}–B_{12} uptake by rat gastric juice in Krebs-Ringer phosphate solution was not affected by rinsing the intestinal segment with aqueous sodium EDTA solution *after* the perfusion. When, however, EDTA was incorporated into the pre-perfusion rinse and into the perfusion fluid containing rat gastric juice, there was abolition of the enhanced uptake of Co^{60}–B_{12}. The use of perfusion and pre-perfusion rinsing fluids containing calcium EDTA again resulted in an enhanced uptake of Co^{60}–B_{12} by the intestinal wall in the presence of rat gastric juice. This radioactivity was also not removed from the half-loops washed with sodium EDTA *after* the perfusion. The use of manganous EDTA during perfusion also allowed enhanced radioactive uptake by the intestine in the presence of rat gastric juice, but a portion of this radioactivity was removed by the subsequent rinsing of the half-loops of intestine with sodium EDTA. The presence of magnesium EDTA in the perfusion fluids resulted in a slight increase of radioactivity by the intestine under the influence of rat gastric juice, but the increase was small and its subsequent elution by sodium EDTA uncertain. In dialysis experiments (not shown) sodium EDTA did not bind vitamin B_{12} against dialysis, and had no effect on the dialyzability of Co^{60}–B_{12} bound to rat stomach extract.

Effect of time of perfusion on uptake of Co^{60}–B_{12}. Paired loops of rat ileum were perfused in single experiments with identical amounts of Krebs-Ringer phosphate solution containing 1.0 ml. of rat gastric juice and 0.015 μg. of Co^{60}–B_{12} but for different periods of time. In the completed series of 27 experiments perfusion times ranged from 4 to 34 minutes. The perfusions were begun at different times and carried out at different rates in each loop so that perfusion of both loops was completed simultaneously. Thereafter each loop was removed, everted and divided in half longitudinally. One half was then rinsed in Krebs-Ringer phosphate solution and the other half in sodium-EDTA solution.

An identical procedure was carried out using 0.5 ml. of pooled human gastric juice (a quantity saturated by 0.015 μg. of Co^{60}–B_{12}). In other experiments 1.0 ml. of human gastric juice (representing an excess of vitamin B_{12} binding power) was used. The effect of time of perfusion and of the subsequent rinsing of one of the half-loops of intestine in Krebs-Ringer phosphate solution and the other half in sodium EDTA solution was determined as in the experiments with rat gastric juice.

Results. Figure 3 demonstrates the linear increase with time of the average amounts of Co^{60}–B_{12} adherent to loops of rat ileum after perfusion with solutions containing rat or human gastric juice, respectively. The values for each experiment, likewise expressed as micromicrograms of vitamin B_{12} and obtained by multiplying the data for radioactivity by a conversion factor,

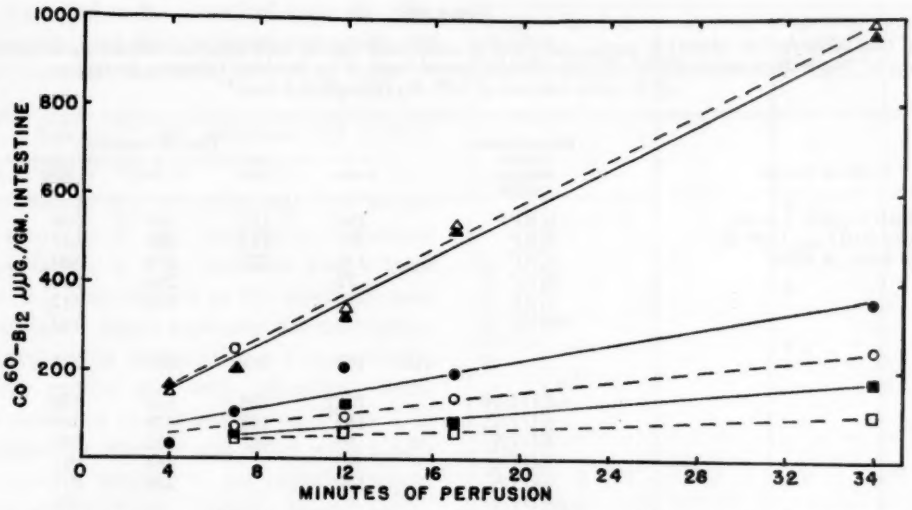

FIG. 3. EFFECT OF TIME OF PERFUSION, OF SPECIES OF GASTRIC JUICE AND OF SUBSEQUENT RINSING WITH DISODIUM ETHYLENEDIAMINETETRAACETATE (EDTA) SOLUTION UPON THE AVERAGE AMOUNTS OF Co^{60}–B_{12} ADSORBED BY PAIRED LOOPS OF RAT INTESTINE

The data from individual experiments upon which the average values plotted in the figure are based are shown in Table III. Solid symbols and solid lines indicate average values after rinsing with Krebs-Ringer phosphate (KRP) solution; open symbols and dashed lines indicate average values after rinsing with EDTA solution. Results obtained with rat gastric juice saturated with Co^{60}–B_{12} are shown by triangles, after similarly saturated human gastric juice by circles, and after half-saturated human gastric juice by squares. It is apparent that the adsorption of Co^{60}–B_{12} increases in a linear fashion with time but more rapidly with rat than with human gastric juice. Note that the Co^{60}–B_{12} adsorbed under the influence of rat gastric juice could not be removed by subsequent rinsing with EDTA solution, whereas with human gastric juice the adsorbed Co^{60}–B_{12} could be partially removed by EDTA solution.

are listed in Table III. In every animal the intestinal loop perfused for the longer interval retained more radioactivity after rinsing than did the loop perfused for a shorter interval. When both kinds of gastric juice were saturated with respect to their vitamin B_{12} binding capacity, the increase of radioactivity was much more rapid in the presence of rat than of human gastric juice. Moreover, when the same amount of Co^{60}–B_{12}, but twice the quantity of human gastric juice was perfused, the increase in radioactivity of the intestinal wall was still slower. Rinsing half of the intestinal loop with sodium EDTA after the experiment had no effect on the residual radioactivity after perfusion with solutions containing rat gastric juice but sharply reduced the radioactivity incorporated under the influence of human gastric juice.

Metabolic requirements of the uptake of Co^{60}–B_{12}. Perfusion studies were carried out using 2.0 ml. of rat gastric juice in Krebs-Ringer phosphate solution in one loop and no gastric juice in the same perfusion fluid in the other. In order to determine the effect of ischemia (or anoxia) on the system, just before the initial pre-perfusion rinse with Krebs-Ringer phosphate solution the vessels at the root of the mesentery of the intestine were ligated.

Similar experiments were performed using loops with intact circulation as usual but with metabolic inhibitors added to the pre-perfusion rinsing and perfusion fluids in both loops. Sodium azide in a concentration of 10^{-2} M was chosen because the animals survived this concentration for the duration of the experiment. When double this concentration of azide was used all of the animals died three to five minutes before the end of the perfusion. Perfusion solutions containing 10^{-2} M sodium cyanide were lethal to most of the rats during the perfusion. In other experiments 10^{-2} M dinitrophenol was employed.

TABLE III

*Effect of time of perfusion, species of gastric juice and of subsequent rinsing with disodium ethylenediaminetetraacetate upon the amount of Co^{60}–B_{12} adsorbed by paired loops of rat intestine following perfusion of the same amount of Co^{60}–B_{12} through each loop**

Perfusion mixture	Postperfusion rinsing solution		Time of perfusion				
			4 min.	7 min.	12 min.	17 min.	34 min.
Rat gastric juice, 1.0 ml., and 0.015 μg. Co^{60}–B_{12} in 4 ml. of KRP	KRP†		286‡	245	490	624	1,480
	KRP		203	143	400	547	1,050
	KRP		132	222	361	300	475
	KRP		78		206	810	840
	KRP		99		146	512	
	KRP					312	
		Av.	160	203	321	518	961
	EDTA§		258‡	208	536	558	1,370
	EDTA		181	136	428	593	1,125
	EDTA		99	407	332	378	482
	EDTA		95		184	915	950
	EDTA		140		234	377	
	EDTA					350	
		Av.	154	250	343	529	982
Human gastric juice, 0.5 ml., and 0.015 μg. Co^{60}–B_{12} in 4 ml. of KRP	KRP		36	51	198	216	333
	KRP		31	43	134	186	357
	KRP			252	366	198	267
	KRP			87	137	176	463
		Av.	34	108	208	194	355
	EDTA		41	31	72	184	229
	EDTA		23	37	92	110	229
	EDTA			155	126	139	194
	EDTA			78	95	120	330
		Av.	32	75	96	138	246
Human gastric juice, 1.0 ml., and 0.015 μg. Co^{60}–B_{12} in 4 ml. of KRP	KRP			43	123	186	179
	KRP			58	202	73	230
	KRP				55	46	122
	KRP					36	159
	KRP						176
		Av.		51	127	85	173
	EDTA			37	15	110	87
	EDTA			49	108	23	134
	EDTA				55	62	149
	EDTA					50	69
	EDTA						73
		Av.		43	59	61	102

* The values shown are for adsorbed Co^{60}–B_{12} (micromicrograms per gram of intestine).
† Krebs-Ringer phosphate solution.
‡ The values for corresponding half-loops of rat intestine after rinsing with KRP and EDTA, respectively, are shown in corresponding positions in each column of figures.
§ Disodium ethylenediaminetetraacetate solution.

Results. As shown in Figure 4, ligation of the mesenteric vessels of the intestinal loop abolished the action of rat gastric juice. On the contrary, the presence of 10^{-2} M sodium azide in the preperfusion rinsing and perfusion fluids did not affect the action of rat gastric juice. Doubling this concentration resulted in death of all the animals during the perfusion period, but nevertheless a significant augmentation of uptake of Co^{60}–B_{12} was produced by the rat gastric juice. Consequently, it was assumed that even at the lower concentration a lethal concentration of azide was

probably presented to the intestinal mucosal cells. Sodium cyanide and dinitrophenol were also not inhibitory (data not shown).

Phase III. Release by the intestinal wall of vitamin B_{12} bound to intrinsic factor

Because vitamin B_{12} in high dosage may be absorbed in man (16, 17, 26) without the assistance of intrinsic factor, it was conceived that release of vitamin B_{12} concentrated at the intestinal wall by intrinsic factor might represent the next physiological step in its assimilation. Consequently, dialysis was carried out with cellophane membranes as described in the section on Phase I in order to determine whether and under what conditions an aqueous extract of rat intestine would make dialyzable Co^{60}–B_{12} initially bound to intrinsic factor.

Equivalence of binding capacities of different species of intrinsic factor preparations. As a preliminary to such experiments it was necessary to determine the amounts of each intrinsic factor preparation possessing similar binding capacities for Co^{60}–B_{12}. Accordingly, increasing amounts of Co^{60}–B_{12} up to 0.06 μg. per ml. were added to rat stomach extract, to human gastric juice and to the hog intrinsic factor preparation when suitably diluted. These preparations were then dialyzed individually in 0.0125 M phosphate buffer at pH 7.0 and 37° C. for five hours. It was assumed that the Co^{60}–B_{12} crossing the cellophane membrane was balanced by an equal quantity of dialyzable vitamin which had not crossed the membrane. The bound vitamin thus was calculated for each sample as the difference between the total concentrations of Co^{60}–B_{12} on opposite sides of the membrane.

Results. In Figure 5 are shown the quantities of Co^{60}–B_{12} bound by 1 ml. of each of the intrinsic factor preparations: rat stomach extract, pooled human gastric juice and the hog intrinsic factor preparation diluted 1 : 16 in water. As may be seen, their binding capacities were approximately equal and increased in direct proportion to the amount of Co^{60}–B_{12} added up to about 0.02 μg. per ml. With larger amounts the primary binding capacity was apparently exceeded and the amounts of Co^{60}–B_{12} bound were distinctly less than the amount added.

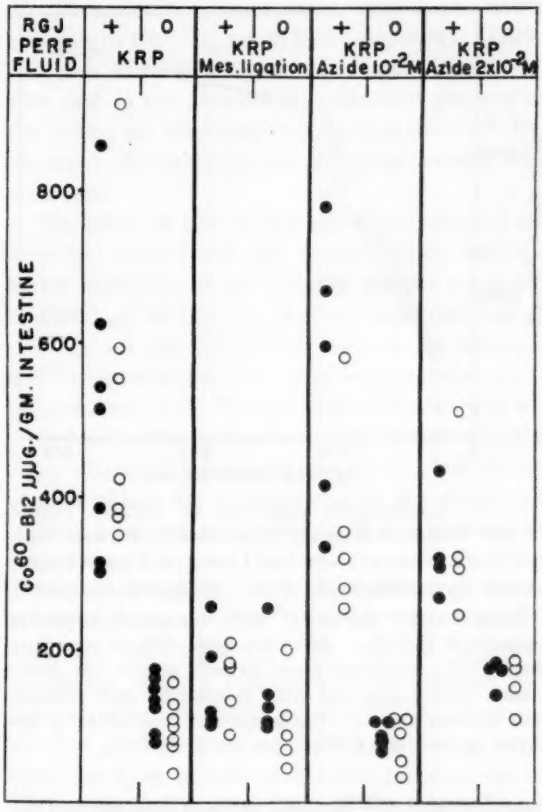

FIG. 4. EFFECT OF LIGATION OF THE MESENTERIC VESSELS, OF SODIUM AZIDE AND OF SUBSEQUENT RINSING WITH DISODIUM ETHYLENEDIAMINETETRAACETATE (EDTA) SOLUTION UPON THE ADSORPTION OF Co^{60}–B_{12} WITH (LEFT-HAND COLUMN) AND WITHOUT (RIGHT-HAND COLUMN) RAT GASTRIC JUICE (RGJ)

Each circle represents the amount of Co^{60}–B_{12} adsorbed by half of each member of a pair of loops of rat intestine. After the perfusion, half of each loop was rinsed in Krebs-Ringer phosphate (KRP) solution (solid circles) and the other half with EDTA solution (open circles). Thus, in each experiment the adsorbed radioactivity of each half of each pair of intestinal loops was measured; in all, four specimens. It is apparent that interruption of the blood supply but not the presence of sodium azide prevents the adsorption of Co^{60}–B_{12} under the influence of rat gastric juice. Once so adsorbed, EDTA solution apparently does not remove it.

Effects of rat intestinal extract on release of Co^{60}–B_{12} bound to different species of intrinsic factor preparations with similar binding capacities. Paired aliquots of rat stomach extract, of human gastric juice and of the diluted hog intrinsic factor preparation, respectively, to which increasing

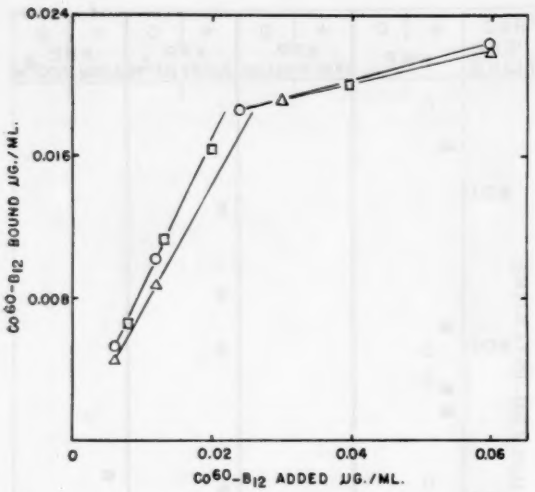

FIG. 5. COMPARATIVE AMOUNTS OF Co^{60}–B_{12} BOUND BY RAT STOMACH EXTRACT (TRIANGLES), HUMAN GASTRIC JUICE (CIRCLES) AND HOG INTRINSIC FACTOR PREPARATION SUSPENDED IN 16 PARTS OF WATER (SQUARES)

To a constant amount of each was added increasing amounts of Co^{60}–B_{12}. After the usual dialysis procedure the Co^{60}–B_{12} remaining bound by each sample was determined. The similar and linear relation for each preparation between the Co^{60}–B_{12} bound and that added is apparent up to about 0.02 µg. per ml. of Co^{60}–B_{12}.

amounts of Co^{60}–B_{12} had been added up to 0.06 µg. per ml. were set up. To one member of each pair was added a constant amount of rat intestinal extract, to the other was added a similar amount of water. Each pair was then dialyzed in 0.0125 M phosphate buffer at pH 7.0 and 37° C. for five hours. The differences between the Co^{60}–B_{12} remaining bound in the individual pairs of dialysis tubes were taken to represent the quantities of Co^{60}–B_{12} made dialyzable by the intestinal extract. In other experiments, Co^{60}–B_{12} was added to the intestinal extract in the absence of intrinsic factor preparations and the binding capacity of the intestinal extract alone was estimated from the quantity of radioactivity that passed the cellophane membrane compared to that migrating in phosphate buffer alone.

Results. As shown in Figure 6, in experiments employing these preparations, the vitamin B_{12} made dialyzable from rat stomach extract by the subsequent addition of equal quantities of rat intestinal extract was 10 times that made dialyzable from human gastric juice. None of the vitamin was consistently made dialyzable from the hog intrinsic factor preparation by rat intestinal extract (data not shown).

The native vitamin B_{12} content of rat intestinal extract, as determined microbiologically, was only 0.025 µg. per ml. Alone it had little or no binding power for added Co^{60}–B_{12} *in vitro*. Thus, 1.0 ml. of the extract was found to allow from 47 to 50 per cent of 0.006 µg. of added Co^{60}–B_{12} to dialyze in five hours at 37° C. A similar result occurred with an equal concentration of Co^{60}–B_{12} in Krebs-Ringer phosphate buffer alone.

Kinetics of the release of Co^{60}–B_{12} from rat stomach extract by rat intestinal extract. In other experiments a constant amount of rat in-

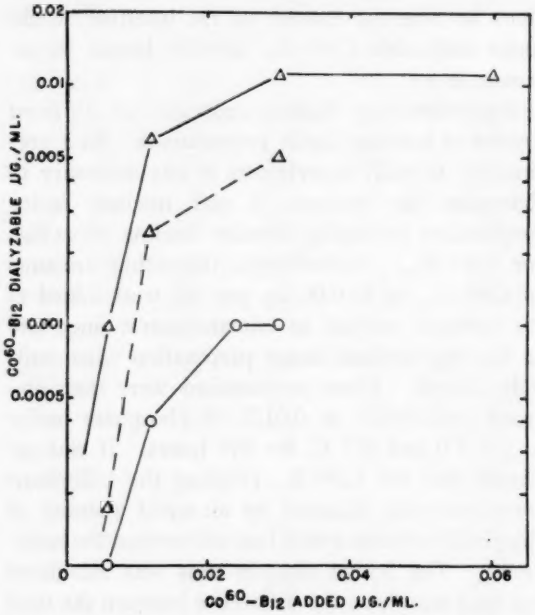

FIG. 6. COMPARATIVE EFFECTS OF A CONSTANT AMOUNT OF RAT INTESTINAL EXTRACT IN RENDERING DIALYZABLE Co^{60}–B_{12} BOUND TO RAT STOMACH EXTRACT (TRIANGLES) AND TO HUMAN GASTRIC JUICE (CIRCLES), RESPECTIVELY

As shown in Figure 5, these preparations as well as a preparation of hog intrinsic factor concentrate had similar binding capacities for Co^{60}–B_{12}. The effect of previously boiled rat intestinal extract upon Co^{60}–B_{12} bound to rat stomach extract is also shown (triangles connected by dashed line). Semilog scales are used. It appears that the same amount of rat intestinal extract is able to render dialyzable about 10 times as much Co^{60}–B_{12} bound to rat stomach extract as when bound to human gastric juice. The intestinal extract was still active after boiling. However, even unboiled extract had no effect upon Co^{60}–B_{12} bound to the hog intrinsic factor preparation (results not shown).

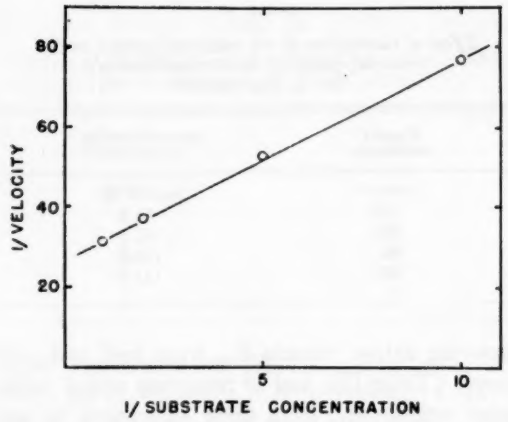

FIG. 7. AMOUNT OF Co^{60}–B_{12} RENDERED DIALYZABLE FROM VARIOUS CONCENTRATIONS OF RAT STOMACH EXTRACT DURING INCUBATION AT 37° C. FOR FIVE HOURS WITH A CONSTANT QUANTITY OF RAT INTESTINAL EXTRACT

The reciprocal of the Co^{60}–B_{12} rendered dialyzable (velocity of the reaction) is plotted on the vertical axis while the reciprocal of the concentration of the rat stomach extract (the substrate) is plotted on the horizontal axis according to the method of Lineweaver and Burk (27). A linear relation is evident.

testinal extract was incubated during the usual dialysis period of five hours at 37° C. with various amounts of rat stomach extract previously saturated with Co^{60}–B_{12}. The quantity of Co^{60}–B_{12} made dialyzable by the intestinal extract was measured.

Results. In Figure 7 are plotted the quantities released from 1.0, 0.5, 0.2 and 0.1 ml., respectively, of rat stomach extract by a constant quantity of rat intestinal extract during five hours under the above conditions. These were arbitrarily taken as a measure of the velocity of the reaction according to the method of Lineweaver and Burk (27). The linear relationship obtained suggests that the five hour incubation interval chosen was probably within the period of constant velocity of the reaction, and also that kinetics of the reaction may be described by the mass law and by the Michaelis-Menten equation. Such kinetics are characteristic of enzyme reactions, but do not prove this is necessarily the case here.

Effect of previous boiling, low pH, and metabolic inhibitors on the activity of rat intestinal extract. Rat intestinal extract was boiled on a water bath for 20 minutes. Then to a constant amount of rat stomach extract were added increasing amounts of Co^{60}–B_{12} up to 0.06 μg. per ml. These mixtures were then dialyzed as usual in the presence and in the absence of a constant amount of the boiled rat intestinal extract and the Co^{60}–B_{12} rendered dialyzable by the intestinal extract was measured.

The effect of pH on the action of unboiled rat intestinal extract was also determined by dialysis. Equal quantities of rat stomach extract saturated by 0.006 μg. of Co^{60}–B_{12} per ml. were dialyzed at pH 3.0, 4.0 and 7.5, respectively, in the presence and in the absence of a constant quantity of rat intestinal extract. The pH of each system was adjusted with 2 N hydrochloric acid before dialysis. After dialysis the amounts of Co^{60}–B_{12} still bound at each pH in the presence and in the absence of intestinal extract were compared as a measure of its activity.

Measured amounts of rat intestinal extract were briefly incubated with 1.2×10^{-2} M mercuric chloride and 1.2×10^{-2} M sodium arsenite. Thereafter, similar quantities of rat stomach extract saturated by 0.006 μg. of Co^{60}–B_{12} were incubated during the usual dialysis period with equal amounts of each of the two preparations of rat intestinal extract containing the metabolic inhibitors as well as with an unmodified sample. The amounts of Co^{60}–B_{12} released by each preparation of rat intestinal extract were determined.

Results. Boiling the rat intestinal extract reduced, but did not abolish, its ability to make dialyzable a portion of the vitamin B_{12} bound to rat stomach extract (Figure 6). Acidification of the system prevented most of the Co^{60}–B_{12} bound by rat stomach extract from being made dialyzable by rat intestinal extract (Table IV). As

TABLE IV

Effect of acidity on the activity of rat intestinal extract in making dialyzable Co^{60}–B_{12} bound to rat stomach extract

Rat stomach extract containing Co^{60}–B_{12} (0.006 μg. per ml.)	Rat intestinal extract	pH	Co^{60}–B_{12} bound after dialysis
			μμg. per ml.
+	0	7.5	4,882
+	+	7.5	1,930
+	+	4.0	3,762
+	+	3.0	4,274

shown in Table V, the presence of mercuric chloride or of sodium arsenite in the quantities used resulted in much of the Co^{60}–B_{12} remaining bound to the rat stomach extract.

Characteristics of the dialyzable radioactivity. The dialysate obtained after incubation of rat stomach extract saturated with Co^{60}–B_{12} together with rat intestinal extract was diluted in 0.05 M phosphate buffer at pH 7.0 and shaken with an equal volume of benzyl alcohol. The partition coefficient of the radioactivity for benzyl alcohol over water was determined as 0.75 to 0.77, which compares closely with that of Co^{60}–B_{12}. This suggests that the dialyzed material is free cyanocobalamin, since different distribution ratios in the solvents would be expected if a fragment of intrinsic factor or other organic substance was bound to the vitamin.

Other characteristics of rat intestinal extract. Rat intestinal extract was incubated with 1 per cent dextran solution for 90 minutes at 37° C. and pH 6.0. The reducing activity of the system (determined as glucose) at 30 minute intervals gradually rose (Table VI). Rat intestinal extract was also incubated with an equal volume of 1 per cent soluble starch solution at pH 6.0 at 37° C. The intensity of the blue color produced by the addition of iodine, as determined at intervals in a Klett-Summerson photoelectric colorimeter with a red filter (No. 1—640 to 700 mμ.), fell rapidly (Table VII).

The intestinal extract thus was capable of hydrolyzing dextran and had amylase-like properties.

TABLE V

Effects of metabolic inhibitors on the activity of rat intestinal extract in making dialyzable Co^{60}–B_{12} bound to rat stomach extract

Rat stomach extract containing Co^{60}–B_{12} (0.006 μg. per ml.)	Rat intestinal extract	Inhibitors	Co^{60}–B_{12} bound after dialysis
			μμg. per ml.
+	0	0	4,280
+	+	0	2,220
+	+	$HgCl_2$	3,720
+	+	$NaAsO_2$	3,340

TABLE VI

Effect of incubation of rat intestinal extract on the reducing power of dextran solution at 37° C. and pH 6.0

Time of incubation	Reducing power (as glucose)
min.	*mg./100 ml.*
0	86.8
30	97.1
60	114.0
90	123.4

DISCUSSION

The dialysis experiments described indicate that human gastric juice is capable of competitively removing native vitamin B_{12} from beef and pork muscle (Table II), and of removing added radioactive vitamin B_{12} from these substances as well as from casein (Table I). Because vitamin B_{12} bound to gastric juice is preferentially absorbed both in pernicious anemia (12) and in the intestinal loop of the gastrectomized rat (13), such binding may constitute an essential initial step in the action of intrinsic factor with respect to the relatively small amounts of vitamin B_{12} in most natural food sources. Because vitamin B_{12} diffuses more readily from food proteins at an acid pH but is bound equally well by human gastric juice or hog gastric mucosal extract (28) at neutral or acid pH values, the presence of normal gastric acidity would appear to be of physiologic advantage in the postulated Phase I of the action of intrinsic factor.

In the rat experiments with paired intestinal loops with intact blood supply, the presence of bivalent cations—especially Ca^{++}—appeared to be necessary for the uptake of vitamin B_{12} by the rat intestine under the influence of rat gastric juice (Figure 2). Thus, rat gastric juice was ineffectual in enhancing the uptake of Co^{60}–B_{12} when Ca^{++} was chelated by EDTA during the perfusion. Because it was shown in dialysis experiments that EDTA neither bound Co^{60}–B_{12} nor affected its dialyzability when bound to rat stomach extract, the effect of EDTA in the intestinal loop must have been on the uptake of Co^{60}–B_{12} already bound to rat intrinsic factor. Chelating the Ca^{++} with EDTA *after* the perfusion did not remove the radioactivity from the intestinal wall. This suggests that the intrinsic factor-bound Co^{60}–B_{12} first adheres to the intestinal wall by means of a calcium-containing bond, but that later this bond to the intestine is dissolved or ren-

TABLE VII

Effect of incubation of rat intestinal extract on soluble starch solution at 37° C. and pH 6.0

Time of incubation	Blue color with iodine
min.	*Klett units*
0	240
3	198
5	14
7	0
9	0

dered unavailable to EDTA. The ease with which the radioactivity may be removed from the intestinal wall when calcium is replaced by manganese suggests that manganese is less efficient than calcium as a bivalent cation in the binding process (Figure 2). This process, so defined, is postulated as Phase II of the action of intrinsic factor.

It is known (29) that a number of mucoproteins contain bonds consisting of $(CaSO_4)^+$ complexes linking amino groups and carboxyl groups, and that this bond may be broken with EDTA. Monovalent cations may replace calcium in this type of bond with marked change in the physical properties of the mucoprotein. Since intrinsic factor is thought to be a mucoprotein (30, 31). this might explain its functional need for bivalent cations. Miller and Hunter (32) and Herbert (25) have demonstrated that hog intrinsic factor will bind Co^{60}–B_{12} to rat liver slices. Moreover, Herbert (25) has found that calcium is required for this effect and that much of this radioactivity may subsequently be removed with EDTA. Calcium has also been shown to be essential for the uptake of Co^{60}–B_{12} by everted segments of rat intestine (14) and by the human intestine (33, 34). Inhibition without complete destruction of human (35) and of rat (13) intrinsic factor activity by low pH has been reported. The marked reduction in ionization of carboxyl groups at low pH would be expected to prevent the formation of the NH_2–SO_4–Ca–OOC complex. With experimentally maintained acidity of the intestinal contents this would occur at the mucosal surface but not within the wall of the living intestine.

If the calcium-dependent bond is accepted as essential to Phase II, the initial reaction between intrinsic factor and the intestinal wall in the presence of Ca^{++}, there still remains to be explained the inability of EDTA to remove Co^{60}–B_{12} bound to the wall only a few minutes after the completion of the perfusion with rat gastric juice. This observation requires that by then the calcium bond is either shielded from the action of EDTA or that the Co^{60}–B_{12} has already been transferred to another type of bond in which bivalent cations are not important. The likelihood of the second possibility becomes apparent from consideration of the action of human gastric juice in the experiments with rat intestinal loops. (Table III and Figure 3.) With human gastric juice the uptake of radioactivity is much slower and a portion of the radioactivity may be removed by subsequent rinsing with EDTA. Moreover, the fraction which cannot be removed by EDTA increases with the duration of the perfusion. Thus, the initial Ca^{++}-dependent bonding may be the essential preliminary step in Phase II, and the change of bond may be the rate-limiting factor in the subsequent absorption of vitamin B_{12} during the hypothetical Phase III of the action of intrinsic factor.

The presence of 2×10^{-2} M sodium azide in the perfusing fluid was sufficient to kill the rat but still allowed an augmented uptake of radioactivity by the intestinal loop under the influence of rat gastric juice. Similar results were obtained with 10^{-2} M dinitrophenol (data not presented). It is probable, therefore, that 10^{-2} M sodium azide, which also caused no inhibition of uptake is sufficient to inhibit respiration in the intestinal mucosal cells. This suggests, as does the activity of hog intrinsic factor in everted sacs of rat intestine at 6° C. (14), that adsorption of vitamin B_{12} to the rat intestine in Phase II is a physical process that is not species-related and that does not require the expenditure of energy. This is, of course, not to say the same of its further transport within the intestinal wall, for which Abels (36) has recently suggested pinocytosis at a molecular level as a possible initial mechanism.

In the dialysis experiments with a crude extract of rat intestine its 10-fold greater ability to release what appears to be free vitamin B_{12} from rat stomach extract compared to its effect on human gastric juice corresponds with the differential effects of rat and of human intrinsic factor

preparations in promoting the assimilation of vitamin B_{12} by the gastrectomized rat (37) and by the rat intestinal loop (Figure 1). Moreover, this species-related activity of rat intestinal extract, if concerned in the conversion of the Ca^{++}-dependent bond might explain the correspondingly greater ability of EDTA to remove a portion of the radioactivity from the intestine after perfusion with human gastric juice (Figure 3). EDTA was similarly effective in Herbert's experiments with a hog intrinsic factor preparation on surviving rat liver slices (25) and everted sacs of rat intestine (15). The total inability of the rat intestinal extract to free vitamin B_{12} from the hog intrinsic factor preparation also resembles the complete failure of hog intrinsic factor preparations to enhance vitamin B_{12} adsorption in either the gastrectomized rat or the rat intestinal loop. For this reason, conclusions concerning the effects of hog intrinsic factor preparations upon rat tissues may be applicable only to certain phases of its physiological action. However, such experiments have disclosed chemical circumstances essential to part of the physiological process (14, 25).

Without the aid of intrinsic factor, absorption of vitamin B_{12} may be accomplished only by using relatively large doses of vitamin B_{12}. Since no convincing evidence exists that a vitamin B_{12}-peptide or other conjugate of vitamin B_{12} with a small molecule is involved in the absorption of vitamin B_{12} (38, 39), Phase III of the action of intrinsic factor may also include the destruction of the intrinsic factor-vitamin B_{12} bond at or just within the intestinal wall, with consequent release of free cyanocobalamin. The vitamin B_{12} thus freed would then either be accepted by an appropriate transport mechanism or would simply diffuse passively into the blood vessels of the intestine.

The active substance (or substances) in the rat intestinal extract reacts with rat stomach extract in a manner which follows the Lineweaver-Burk (27) variation of the Michaelis-Menten equation. This is compatible with enzymic activity, but does not prove that such is the case. The intestinal extract is partially inactivated by heat and hydrolyzes both starch and dextran. It may be identical with the dextran hydrolyzing enzyme described by Androuny, Bloom and Wilhelmi (40) or with the 1,6-glycosidase described by Larner and McNickle (41). No evidence, however, is available to prove that the active principle in this rat intestinal extract necessarily has any physiological significance with respect to vitamin B_{12} absorption. It is interesting, however, that it exhibits a species-related activity similar to that of intrinsic factor in the rat.

SUMMARY

1. Human gastric juice competitively removes native vitamin B_{12} or added Co^{60}–B_{12} from pork or beef muscle preparations during dialysis *in vitro*. This process is favored by low pH because such food proteins, as well as casein, lose vitamin B_{12} more readily at low pH values, whereas human gastric juice loses none of its native vitamin B_{12} when dialyzed against water at either pH 6.0 or pH 1.5.

2. There is a linear increase with time in the quantity of Co^{60}–B_{12} on or in the wall of loops of rat ileum with intact circulation after perfusion with rat or human intrinsic factor in the presence of Krebs-Ringer phosphate solution. This increase is more rapid in the presence of rat stomach extract than of human gastric juice. No certain effect was noted with a hog intrinsic factor preparation.

3. This action of preparations containing rat or human intrinsic factor is not affected by the presence of 2×10^{-2} M sodium azide, but is prevented by occluding the blood supply of the intestine. It is also prevented by the presence of sodium ethylenediaminetetraacetate (EDTA) *during* the perfusion but not by the presence of calcium EDTA. That the dialyzability of Co^{60}–B_{12} alone or when bound to rat stomach extract is unaffected by sodium EDTA indicates that calcium is involved in the binding of intrinsic factor to the intestinal mucosa. Manganese or magnesium only partially neutralizes the inhibitory effect of sodium EDTA. A portion of the vitamin B_{12} adsorbed by the rat intestine during perfusion with Krebs-Ringer phosphate in the presence of human gastric juice, but none of that adsorbed in the presence of rat stomach extract, is removed *after* the perfusion by rinsing with sodium EDTA.

4. A crude extract of rat intestine is capable of

making dialyzable, apparently as free vitamin, a portion of the Co^{60}–B_{12} bound to rat stomach extract, much less of that bound to human gastric juice, and none of that bound to a hog intrinsic factor preparation. Its action is partially inhibited by boiling, by low pH and by mercuric chloride and sodium arsenite. The intestinal extract hydrolyzes both dextran and starch.

5. A hypothesis is presented to explain the observed differences in activity of different species of intrinsic factor in the absorption of vitamin B_{12} by the gastrectomized rat. The present observations suggest that three phases can be recognized in the action of intrinsic factor in the rat: Phase I, a non-species-related competitive binding by intrinsic factor of dietary vitamin B_{12}; Phase II, a non-energy-requiring adsorption of intrinsic factor-bound vitamin B_{12} by the intestinal mucosa involving a bivalent cationic dependent bond; and Phase III, a species-related conversion of the Ca^{++}-dependent bond and release of free vitamin B_{12} at the surface of or within the intestinal wall, possibly as a result of intestinal enzymic action.

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THE EFFECT OF EXERCISE ON THE CONCENTRATION AND TURNOVER OF PLASMA NONESTERIFIED FATTY ACIDS *

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Recent work has suggested that the albumin-bound fatty acids of blood (herein called FFA, the free or nonesterified fatty acids of blood) (1) might be oxidized directly by muscle. If this is so, and if this material is utilized promptly, either directly or through an intermediate, acute changes would be expected to take place in blood during increased physical activity. Such behavior would be particularly expected because fatty acids are known to turn over rapidly and to circulate in low concentration. Therefore a study was undertaken to investigate changes in plasma fatty acid during exercise and to evaluate the direction and magnitude of these changes.

Few human experiments on this subject have been published and little information is available about fatty acid utilization by skeletal muscle. Andres, Cader and Zierler (2) noted that the amount of glucose taken up by the human forearm is less than equivalent to the local oxygen consumption and CO_2 production. Furthermore, their measurements of forearm R.Q. hinted at relatively extensive consumption of lipids or lipid products. Gordon has shown that the human forearm usually (but not always) takes up more fatty acid than it releases, suggesting that these fatty acids are taken up directly by muscle cells (3). Interpretation of this type of measurement with respect to direct utilization of a substrate is difficult since net arteriovenous differences reflect the sum of a number of possible effects such as deposition into fat depots, release from fat depots and uptake by muscle. The *in vitro* utilization of fatty acid by skeletal muscle at rest and during electrical stimulation has recently been demonstrated by Fritz and co-workers who have, in addition, shown inhibition of the reaction with increasing concentration of albumin in the medium (4, 5).

The work of Fredrickson and Gordon, and Bierman and colleagues (6, 7) on the disappearance from the vascular compartment of injected carboxyl-labeled, albumin-bound fatty acid is pertinent to the present discussion since similar techniques have been applied in the present investigation.

METHODS

The study was carried out in two parts: first, the effect of exercise on arterial plasma FFA level; and second, the effect of exercise on the disappearance of injected, prepared albumin-bound radiopalmitate.

It was found that lactic acid generated during exercise caused considerable spurious elevation of FFA as determined by the Dole method (1). For this reason a modified Dole procedure was used, consisting of washing the organic solvent layer with 0.02 per cent sulfuric acid.[1] The method and its validation will be described in detail elsewhere (8).

Triglycerides were determined by the method of Van Handel and Zilversmit (9), glucose by the iodometric titration of Somogyi (10), and lactic acid by the method of Barker and Summerson (11). Plasma lipolytic ac-

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[1] In the organic solvent phase of the Dole procedure 1.5 to 2.5 per cent of lactic acid of plasma equilibrates. This means that every milligram per 100 ml. increase in lactic acid of plasma would be expected to increase the apparent FFA titration by about 2 μMoles per L. This theoretical increase correlates well with actual differences in "washed" and "unwashed" FFA titrations in exercising individuals and with values simultaneously obtained by the Gordon and Cherkes method. The modified procedure was found to yield 91 per cent recovery of fatty acid.

tivity was determined by an adaptation of a method by Engelberg (12–14). This adaptation measures the release of fatty acid from Ediol® (a fat emulsion) incubated with plasma.

Measurement of the activity of plasma after injection of radiopalmitate was carried out in the following manner. Two ml. samples of plasma were extracted as in the Dole procedure for determination of FFA. Eight ml. aliquots from the organic solvent phase, which represent the fatty acids from 1.78 ml. plasma, were placed in separate tubes. In order to separate fatty acids from neutral lipid [2] (15), 8 ml. of 0.1 N NaOH in 50 per cent ethanol was added to the tubes to convert the fatty acids to the sodium salt and extract them into an aqueous phase. After removal of the upper phase containing neutral fats, the sodium palmitate in the aqueous layer was acidified to a pink end point by addition of 0.25 N HCl using thymol blue indicator. The fatty acids were then re-extracted with heptane and the heptane layer was transferred to 50 ml. beakers. This was accomplished by removing as much hepatane as possible and then washing twice with more heptane and transferring the residue. After partial evaporation of heptane, the remaining heptane and fatty acid were transferred quantitatively to deep stainless steel planchets, 2.5 cm. in diameter. Ninety-nine per cent isopropyl alcohol was used for final washing and quantitative transfer from the beakers to the planchets. The fatty acids were dried under a stream of cool air at room temperature and counted as soon as possible in a windowless gas flow counter. With the amount of residual material on the planchets, self-absorption was considered negligible. Planchets were stored in a freezer to minimize sublimation. After several days of storage, no detectable loss of activity was noted, probably because of the presence of sufficient endogenous fatty acid. When subjected to the above procedure, 88.5 per cent of palmitic acid-1-C^{14} added to plasma was recovered. Duplicate extractions showed a maximum variation of 1.6 per cent from the mean.

Albumin-bound palmitic acid-1-C^{14} was prepared by dissolving the acid in methyl ethyl ketone and converting to the potassium salt by means of a slight excess of alcoholic potassium hydroxide. Three ml. of physiologic saline was added and the solution was evaporated over a water bath until the odors of alcohol and methyl ethyl ketone were no longer detectable. Equivalent quantities of human albumin were added and the clear solution was sterilized by passage through an ultrafine bacterial filter.

In handling the data concerning disappearance of radiopalmitate, the following assumptions were made: first, during the period of exercise considered in this study, there is no consistent or significant change in plasma FFA concentration following the initial fall (Table I); second, the initial part of the curve representing the semilogarithmic plot of the disappearance of radiopalmitate lies sufficiently close to a straight line during both rest and exercise to permit useful estimates of FFA transfer rates based on equations describing such straight line relationships; third, in the steady state and with constant transfer rates for the unlabeled substance, the observable behavior of a tracer is independent of the reaction order (16–18) and the transfer equation has the form of an equation describing a first order reaction (19). The departure from linearity could be the result of a number of possible factors such as recirculation of label (6) or differences in the rate of release of FFA from various binding sites on albumin (20). Although a rise in plasma FFA concentration could account for the apparent decrease in the rate of radiopalmitate disappearance, this is not the case here, since a decrease always occurred regardless of whether FFA concentration rose, fell, or remained the same either during rest or after the initial fall caused by exercise.

TABLE I

Arterial FFA concentrations in millimoles per liter obtained at first and tenth minutes after injection of radiopalmitate during rest and exercise

Subject	Rest		Exercise	
	First min.	Tenth min.	First min.	Tenth min.
CK	0.67	0.66	0.56	0.43
MJ	0.76	0.96	0.60	0.54
RB	1.07	1.16	0.77	0.83
RW	1.24	1.18	0.79	0.97
RP	0.42	0.33	0.31	0.33
WF	0.69	0.60	0.54	0.60

Assuming constancy of flux of unlabeled FFA into and out of plasma: $dx = -xF/W\ dt$ where x equals the amount of label in plasma; t, the time in minutes; W, the amount of unlabeled fatty acid in plasma; and F, the number of millimoles of fatty acid entering or leaving the vascular compartment per minute. Then, $x = x_o e^{-Ft/W}$. Since F/W is constant and equals the fraction of the total fatty acid in the vascular compartment leaving per minute, let $F/W = K$. Then, $x = x_o e^{-Kt}$ (19, 21).

The slope of the disappearance curve for radiopalmitate from the second to the fourth minute after injection was used to establish the value "K," as in the studies of Fredrickson and Gordon (6). This was done because the region usually represents the first clear straight line after an interval of time in which mixing in the blood is reasonably certain to be complete. Though this is generally true of curves obtained in resting subjects, there is reservation concerning the validity of this choice where exercise curves are concerned, so "K" was also calculated from the one to two minute slope. These reservations will be discussed at a later point. A FFA concentration obtained one minute after injection of label was used to estimate total plasma FFA, W, in the calculation of flux, assuming a plasma volume of 3.5 L.

[2] Data by Fredrickson and Gordon (6), confirmed in this laboratory, indicate that there is considerable conversion of FFA to neutral fats and that the latter must be removed for accurate determination of palmitic acid-1-C^{14} activity.

TABLE II

*The effect of exercise on plasma FFA concentration**

	M. R.	B. L.	E. B.	J. O.	G. M.	P. B.	G. W.	Mean
First control	0.72	1.12	0.75	0.96	0.61	1.08	0.69	0.85
Second control	0.75	1.06	0.74	0.97	0.75	1.12	0.73	0.87
After 7 min. exercise	0.66	0.76	0.51	0.78	0.47	0.72	0.53	0.63
After 15 min. exercise	0.69	0.96	0.48	0.68	0.45	0.71	0.52	0.64
After 15 min. rest	0.98	1.20	0.94	1.04	0.99	1.29	0.69	1.02

* FFA concentration is expressed in millimoles per liter.

EXPERIMENTAL

First experiment. Seven healthy male volunteers between the ages of 20 and 25 were selected. These subjects were permitted a 600 calorie, low fat, high carbohydrate breakfast and were then fasted for six hours before and throughout the procedure. A Cournand needle was positioned in a brachial artery in each subject. After a 15 minute rest period, subjects exercised vigorously for 15 minutes by pedaling a stationary bicycle. The subjects were instructed to exercise as vigorously as possible, but to pace themselves so that they would be able to continue for 12 to 15 minutes. Blood samples were drawn in heparinized syringes during all phases of the procedure for determination of blood fatty acid, glucose, triglyceride, lactic acid and lipoprotein lipase activity.

Second experiment. Another group of eight healthy male volunteers between the ages of 21 and 25 was given 0.002 mc. of prepared albumin-bound palmitic acid-1-C^{14} intravenously while in the resting state, and brachial artery blood samples were drawn every minute for 10 minutes.

From 30 to 35 minutes after the resting samples were obtained the subjects began vigorously pedaling a stationary bicycle. After two to three minutes of exercise, another 0.002 mc. of palmitic acid-1-C^{14} was given and the sampling repeated as in the control period, but with the subjects maintaining vigorous activity.

Plasma samples were analyzed for FFA concentration and, in addition, were extracted as previously outlined for measurement of palmitic acid-1-C^{14} activity. Semilogarithmic plots of plasma palmitic acid-1-C^{14} activity were used to measure the disappearance rate of the radioisotope from the vascular compartment.

RESULTS

The effect of exercise on blood FFA level

The results (Table II) reveal consistent, fairly large and rapid falls in the level of arterial FFA during exercise. These effects are best demonstrated graphically (Figure 1). The average control level of 0.87 mMole per L. fell to 0.64 mMole per L. during exercise, a drop of 26.4 per cent ($p < 0.01$). After a 15 minute rest period, the average level rose to 1.02 mMoles per L., a level significantly greater than the control values. No significant changes related to exercise were noted in blood glucose, triglyceride or serum lipoprotein lipase activity. Blood lactic acid was elevated considerably during exercise, rising from 10.9 to 68.9 mg. per 100 ml. and from 7.2 to 67 mg. per 100 ml. in the two subjects in whom measurements were made. Furthermore, during the short period of exercise studied, the lowered FFA levels remained stabilized (Table I) until the physical activity was stopped, after which fatty acid concentration rose to levels above those of the control period ($p < 0.01$).

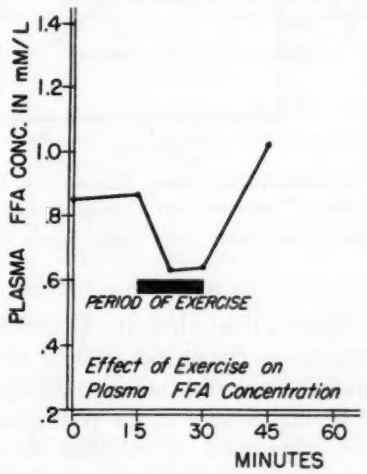

FIG. 1. THE EFFECT OF EXERCISE ON PLASMA FFA CONCENTRATION

Effect of exercise on the disappearance of palmitic acid-1-C^{14} from blood

In the eight subjects studied, the initial rate of disappearance of 0.002 mc. of palmitic acid-1-C^{14} was more rapid during exercise than at rest. This can be seen by inspection of the graph of mean

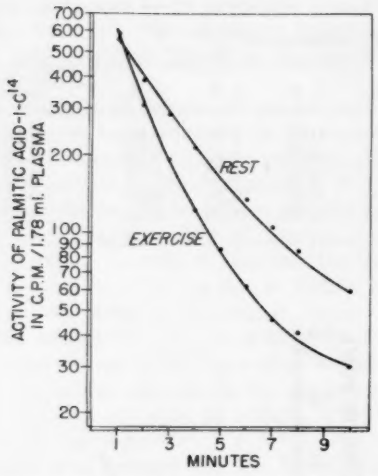

FIG. 2. DISAPPEARANCE FROM PLASMA OF PALMITIC ACID-1-C^{14} AT REST AND DURING EXERCISE

The curves are drawn from mean values from eight subjects.

values (Figure 2), in which the logarithms of the concentrations in counts per minute are plotted against time. The values obtained during the second and fourth minutes of the resting and exercise curve were used to calculate the constant "K" (Table III), representing the fractional rate of disappearance of FFA per minute, *i.e.*, the fraction of the amount of FFA present in the blood disappearing per minute (6) (*vide supra*). During the second to fourth minutes, the mean value for "K" was 0.294 per minute at rest and 0.473 per minute during exercise, an increase of 61 per cent. This would mean that the mean half-life of plasma FFA during the specified time changed from 2.36 to 1.46 minutes.

DISCUSSION

The data indicate that the amount of exercise performed promptly lowered plasma FFA levels. This lowering was rapid, and maximum in the first specimen drawn at seven to eight minutes after exercise was begun. In addition, an equally rapid rise above the pre-exercise level occurred upon cessation of exercise.

The above data show that exercise lowers FFA, but do not reveal whether this is due to an accelerated removal from or a decreased entry into the vascular compartment. This problem is resolved by the second part of the study which shows that exogenous palmitic acid-1-C^{14} is re-

TABLE III

Calculated flux, calculated fractional disappearance rates of plasma FFA during rest and exercise, and data used for calculations

Subject		cpm at 1 min.	cpm at 2 min.	cpm at 4 min.	FFA conc. at 1 min. in mM./L.	K 2–4 min.	2–4 min. Flux/L. plasma	K 1–2 min.	1–2 min. Flux/L. plasma
M. J.	Rest	703	443	240	0.76	0.307	0.23	0.460	0.35
	Ex.	799	409	167	0.60	0.437	0.26	0.668	0.40
R. B.	Rest	448	335	200	1.07	0.256	0.28	0.290	0.31
	Ex.	577	291	105	0.77	0.512	0.39	0.687	0.53
R. P.	Rest	661	356	166	0.36	0.382	0.14	0.619	0.22
	Ex.	516	193	68	0.31	0.522	0.16	0.987	0.31
R. W.	Rest	445	386	223	1.24	0.274	0.34	0.143	0.18
	Ex.	439	223	82	0.84	0.500	0 42	0.677	0.52
C. K.	Rest	510	318	187	0.67	0.266	0.18	0.472	0.32
	Ex.	448	265	110	0.50	0.444	0.22	0.525	0.26
J. R.	Rest	561	430	235		0.302		0.265	
	Ex.	690	423	180		0.427		0.488	
L. S.	Rest	492	365	243		0.203		0.299	
	Ex.	550	332	145		0.414		0.504	
W. F.	Rest	634	467	228	0.69	0.358	0.25	0.306	0.21
	Ex.	669	342	117	0.56	0.536	0.30	0.670	0.38
Mean	Rest	557	388	276		0.294	0.248	0.356	0.265
	Ex.	587	310	192		0.473	0.301	0.650	0.398

moved more rapidly during exercise. Since Fredrickson and Gordon have pointed out that the removal of labeled palmitic acid is qualitatively identical with the removal of most other long chain fatty acids, it is concluded that the early effect of exercise is to lower plasma FFA by accelerating its removal from blood.

Flux, or the amount of FFA entering and leaving the vascular compartment per minute, as calculated from the two to four minute values of "K," was 0.175 mMole per minute higher during exercise, an increase of 25 per cent ($p < 0.02$).

Inspection of the mean exercise and resting curves (Figure 2) shows that the rate of disappearance of label during exercise is more rapid during Minutes one to two than subsequently. If one assumes that mixing is complete at one minute and uses the one to two minute values for calculation of "K," the "K's" for rest and exercise then become 0.356 and 0.650 per minute, respectively, a difference of 82 per cent ($p < 0.01$); and since plasma FFA is unchanged, the calculated differences in flux would be greater than those calculated above (45 per cent). Since the earliest values after mixing is complete are more likely to represent the true disappearance rate of label from plasma, there is some virtue in choosing these points instead of the two and four minute points as others have done in such calculations (6). Lack of knowledge of the time of complete mixing makes it impossible to choose the proper portions of the curves with certainty,[3] nevertheless, regardless of the choice, an increase in flux both into and out of plasma exists.

The rise in plasma FFA after exercise is of interest and could mean that "K" suddenly returns to a lower level while mobilization factors are still operating at an accelerated rate.

Fredrickson and Gordon (6) have shown that "K" varies inversely with plasma fatty acid concentration in the resting individual at levels below 0.6 to 0.8 mMole FFA per L. The resting

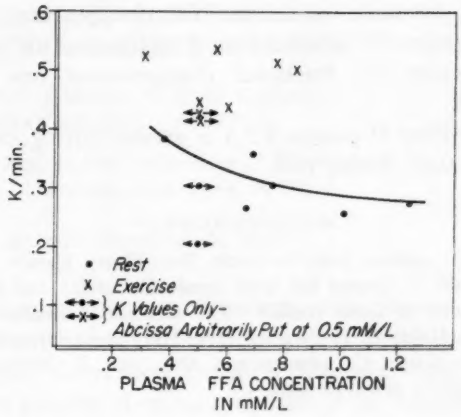

FIG. 3. THE RELATIONSHIP OF PLASMA FFA CONCENTRATION TO THE FRACTIONAL DISAPPEARANCE RATE AT REST AND DURING EXERCISE

data as seen in Figure 3 are consistent with this observation, but the changes in "K" brought about by exercise are outside this range and cannot be accounted for merely on the basis of the decrease in FFA concentration.

The possible effect of lactic acid production during exercise on the level of plasma FFA is raised by the above data. Since increased blood concentration of this substrate persists for much longer than the decrease in plasma fatty acid (23) it is felt that there is probably no immediate relationship between the two and that elevated lactic acid is not responsible for the decline in plasma FFA concentration. In a single experiment, infusion of sodium lactate had no effect on plasma FFA.

The data presented do not determine the fate of the removed FFA. However, unpublished studies by Fredrickson (24) show a five- to sixfold increase in $C^{14}O_2$ excretion during exercise. This and similar problems in FFA utilization in relation to exercise are being examined currently in our laboratory.

SUMMARY AND CONCLUSIONS

1. The relationship between exercise and plasma fatty acid turnover was studied by observing the rate of disappearance of palmitic acid-1-C^{14} and by studying changes in plasma FFA concentration.

2. Acute exercise lowers plasma FFA concentration. There is a significant rise in plasma FFA concentration immediately after exercise.

[3] Experiments with Evans blue dye indicate that mixing is complete between 90 to 120 seconds (22). However, only minor fluctuations in concentration occur after 30 seconds. It is felt, in relation to the present investigation, that there is more error in the first minute samples but that this error is not great at rest and that it is smaller during exercise.

3. Exercise accelerates the disappearance of administered albumin-bound radiopalmitate and increases the fractional disappearance rate of FFA.

4. Flux of plasma FFA is greater during exercise than during rest.

ACKNOWLEDGMENT

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THE DIAGNOSIS OF PHEOCHROMOCYTOMA BY DETERMINATION OF URINARY 3-METHOXY,4-HYDROXYMANDELIC ACID *†

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In 1957, Armstrong, McMillan and Shaw demonstrated a metabolite of epinephrine (E) and norepinephrine (NE) in normal human urine (2, 3). This substance, 3-methoxy,4-hydroxymandelic acid (VMA, for vanillylmandelic acid), was excreted in abnormally large amounts in three patients with pheochromocytomas (3). Recent studies of sympathomimetic amine metabolism (Figure 1) (4–7) suggested that the urinary excretion of the degradation products of E and NE, such as their 3-methylated derivatives (M and NM respectively) and VMA, might exceed E and NE excretion by some 10- or 20-fold. The diagnosis of pheochromocytoma might therefore be facilitated by analysis of urine for these phenolic compounds.

Armstrong, Shaw and Wall's chromatographic technique for determination of urinary VMA (8, 9) was modified and used for the quantitative determination of VMA excretion in the urines of 15 normal subjects, 36 patients with primary hypertension, and 30 subjects with pheochromocytomas. Correlative E and/or NE determinations were performed on most of these urine samples.

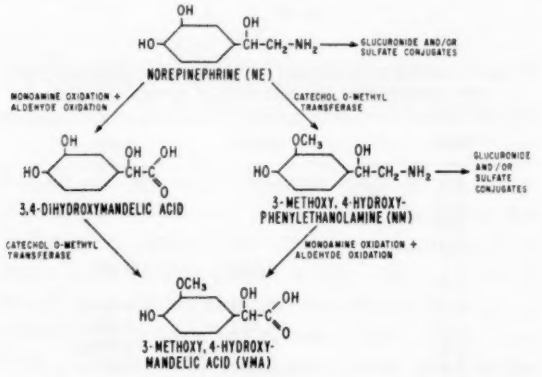

FIG. 1. MAJOR METABOLIC PATHWAYS OF NOREPINEPHRINE

METHODS

VMA determination. Patients receiving no medications and ingesting no fruit, coffee, tea and substances containing appreciable quantities of vanilla for a period of 24 hours, were requested to collect a urine specimen prior to breakfast. An aliquot of urine equivalent to 0.5 mg. of creatinine (technique of Jaffé) was placed in a 15 ml. graduated centrifuge tube, the volume brought up to 2 ml. with water, the pH brought to 2 with a few drops of 3 N HCl, and the tube placed in a boiling water bath for 10 minutes. Following this hydrolysis, the pH was brought to 0.5 to 1.0 with 3 N HCl, 4 ml. of ethyl acetate was added and the tube shaken and centrifuged. The ethyl acetate was removed with a capillary pipette (being careful to avoid aqueous droplets) and the procedure repeated twice with 2 ml. of ethyl acetate. The organic extract was combined in another 15 ml. centrifuge tube, placed in a water bath at 40 to 50° C., and blown to dryness with a stream of air. The sides of the tube were then rinsed with 0.5 ml. absolute ethanol and the contents blown to dryness again. The residue was dissolved in ethanol or ethyl acetate for spotting about 1.5 inches from the corner of a 1 foot square of Whatman No. 1 filter paper. The residue was taken up in a second or third volume of organic solvent in order to insure quantitative transfer to the filter paper. However, the volumes remained small and the spotting procedure was performed slowly enough to keep the spot less than 7 to 8 mm. in diameter. Similar spotting of appropriate quantities of a VMA [1] standard, about six inches above the unknown spot (Figure 2), permitted quantitative estimation of VMA in the aliquot. The filter paper was then stapled in the form of a cylinder, placed in a 6 × 18 inch glass cylinder (possessing a ground glass top which could be sealed by application of silicone grease and a plate glass cover) to which 80 ml. of isopropanol: water: concentrated NH_4OH (40:9:1) solvent system had been added. About 15 hours later, when the solvent front

* This work was aided by grants from the American Heart Association and the National Heart Institute (H 1164), Bethesda, Md.

† A preliminary communication of this work has appeared previously (1).

[1] Kindly supplied by Dr. R. W. Schayer, Merck & Co., Rahway, N. J.

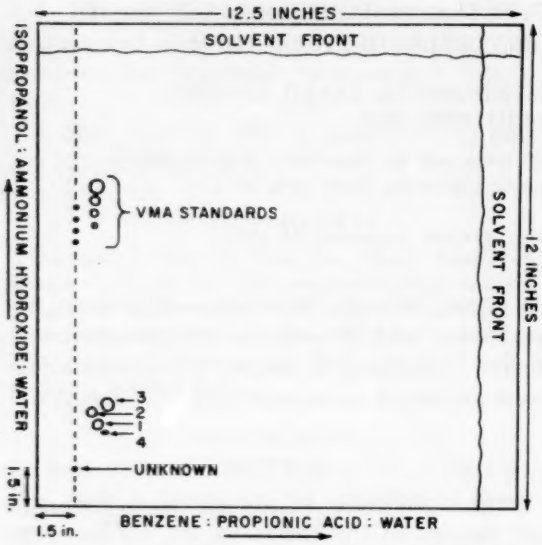

FIG. 2. SAMPLE VANILLYLMANDELIC ACID (VMA) CHROMATOGRAM

1, VMA = purple; 2, *m*-hydroxyhippuric acid (8) and *p*-hydroxymandelic acid (9) = salmon; 3, *m*-hydroxyphenylhydracrylic acid (9) = rose; 4, 5-hydroxyindoleacetic acid = brown.

had run 25 to 30 cm., the paper was removed, allowed to dry, and restapled so as to permit chromatography at a 90° angle to the first run in a solvent system of benzene: propionic acid: water (20:14:1). The paper could be removed in about 3.5 hours (25 to 30 cm. movement of the front). It was allowed to dry in the air overnight and then sprayed with diazotized *p*-nitroaniline. The latter was prepared by dissolving 0.5 Gm. of *p*-nitroaniline in a solution of 10 ml. of concentrated HCl in 490 ml. of water and mixing with equal volumes of 0.2 per cent (w/v) $NaNO_2$ prior to use. Immediately before spraying, this diazotized *p*-nitroaniline was mixed with an equal volume of 10 per cent K_2CO_3. VMA appeared as a purple spot with an R_f of 0.27 (isopropanol: NH_3) and 0.15 (benzene: propionic acid).[2] Densitometric and subjective comparison of the unknown with VMA standards simultaneously spotted on each chromatogram yielded a reproducible accuracy of ± 10 per cent and a sensitivity of 0.2 to 0.3 μg. Diminishing the urine aliquot to 0.25 mg. creatinine or less improved the accuracy in samples high in VMA content.

E and NE urine analyses were performed within a few days of collection by the techniques of von Euler and Floding (10) and Goldenberg, Serlin, Edwards and Rapport (11), after which the acidified samples from patients with pheochromocytomas were kept at 10° C. for from 3 to 42 months prior to analysis for VMA.

[2] The authors are indebted to Dr. M. D. Armstrong for his instruction in this method (9).

Each patient with pheochromocytoma was surgically proven by removal of the tumor with consequent cure of the hypertension. The patients suffering from primary or essential hypertension fulfilled the usual diagnostic clinical criteria and possessed none of the stigmata of Cushing's syndrome, chronic renal disease, pheochromocytoma, primary aldosteronism, or coarctation of the aorta. These patients had Grade I to III changes in their fundi and none was classified as "malignant" hypertensive. Although some had enlarged hearts and electrocardiographic changes, none had congestive heart failure or azotemia. Two patients with pathologically proven carcinoid syndrome and one with hypertension secondary to chronic renal disease were studied. The effects of phenylephrine, ephedrine, naphazoline and isopropylarterenol on VMA excretion were studied on three normal subjects.

RESULTS

The urinary VMA excretion of normal subjects varied from 0.8 to 2.0 μg. per mg. of creatinine (Table I). The NE content averaged but 1 per cent of the VMA.

The urinary VMA excretion of the patients suffering from primary hypertension (Table II) varied from 0.7 to 3.0 μg. per mg. creatinine, or approximately 100 times the NE content of these samples. The lowest figure (0.5 μg. of VMA per mg. creatinine) occurred in a specimen from a patient suffering from hypertension secondary to chronic renal disease with azotemia.

The urinary VMA excretion of patients with pheochromocytomata (Table III) varied from 6.0

TABLE I

Urinary vanillylmandelic acid (VMA), norepinephrine (NE) and epinephrine (E) excretion of normal subjects

	VMA*	NE*†	E*†
	1.0		
	1.3		
	0.8		
	1.8		
	1.2	0.016	0.003
	2.0		
	1.5	0.038	0.005
	1.7	0.012	0.003
	1.2	0.002	0.0005
	1.3	0.004	0
	1.2	0.014	0.001
	1.3	0.002	0.001
	1.2		
	2.0		
	1.0		
Mean	1.4 (S.D. = 0.38)	0.013	0.002

* Micrograms per milligram creatinine.
† Technique of von Euler and Floding (10).

TABLE II

Urinary vanillylmandelic acid (VMA), norepinephrine (NE) and epinephrine (E) excretion of patients with primary hypertension

	VMA*	NE*†	E*†
	1.3		
	1.8		
	2.0		
	1.0		
	2.5		
	2.5	0.009	0.003
	1.0		
	1.2	0.008	0.001
	1.0		
	1.2		
	0.8		
	1.3	0.017	0.001
	2.5		
	1.2		
	3.0	0.002	0.003
	1.2		
	0.7		
	1.0	0.009	0.001
	1.0	0.018	0.003
	1.5	0.022	0
	1.0	0.016	0.001
	1.3		
	1.5		
	1.2		
	1.3		
	1.0		
	1.5		
	2.5		
	1.0		
	1.0		
	1.0		
	0.8		
	1.0		
	1.7		
	1.3		
	2.5		
Mean	1.4 (S.D. = 0.6)	0.012	0.002
	0.5‡	0.006	0

* Micrograms per milligram creatinine.
† Technique of von Euler and Floding (10).
‡ Hypertension secondary to chronic renal disease with uremia.

to 40 μg. per mg. creatinine (4.4 to 54.7 mg. VMA per 24 hours). The VMA exceeded the NE plus E content (per milligram creatinine) by some 3- to 94-fold (mean = 10). No correlation could be made between the duration of storage of pheochromocytoma urines and their VMA content. Urinary VMA has been found to be stable for many months when kept at pH 3 to 4 at 10° C. Moreover, standard solutions of VMA have remained stable for over a year when kept in this manner.

Two patients with carcinoid syndrome revealed extremely large 5-hydroxyindole acetic acid spots (Figure 2) on the chromatograms, permitting immediate suspicion of the urines as those of patients with carcinoid.

A normal subject given 10 mg. of phenylephrine, 10 mg. of ephedrine, and 0.8 mg. of naphazoline by means of nose drops excreted 1.3 μg. of VMA per mg. creatinine following administration of each of these drugs. When 4 mg. of isopropylarterenol was administered by inhalation and when 37.5 mg. was given sublingually, the VMA excretion of two normal subjects remained at control levels.

DISCUSSION

Previous studies demonstrated that no more than 1.5 to 4 per cent of parenterally administered NE appeared unchanged in the urine (11, 12). More recently Goodall, Kirshner and Rosen (13) confirmed these findings by recovering only 4 per cent of parenterally administered NE-2-C^{14} in the unchanged form in the urine. On the other hand, VMA has been variously ascribed to account for 27 to 36 per cent of the urinary excretion of isotopically-labeled NE (13) and E (5, 7). The results of this study confirm the fact that normal as well as hypertensive subjects excrete many times as much VMA as NE in their urine. Increased excretion of NM, the only other NE metabolite believed to occur in urine in large quantities (6, 13), has also been suggested as a possible means of diagnosing pheochromocytoma (6). Up to this time, however, the procedures for demonstrating NM and M in urine have been inadequate for routine use (14).

The determination of urinary VMA in this study afforded differentiation of normal subjects from those with pheochromocytomas in every instance. Armstrong (9) has drawn attention to the fact that other conditions, such as shock, may be associated with abnormally high VMA excretion. This possibility does not limit the usefulness of this test since its application will be predominantly in the differentiation of primary hypertension from hypertension due to pheochromocytoma. That it serves the latter function is clear since no overlapping occurred between these groups in this study.

Urinary NE excretion of hypertensive patients was previously found to be slightly elevated (11, 12), reduced (15), and normal (16). It is there-

TABLE III

Urinary vanillylmandelic acid (VMA), norepinephrine (NE) and epinephrine (E) excretion of patients with pheochromocytomata

Patient	Date of collection	VMA*	NE*	E*	VMA†	NE†	E†
1	12/55	25	4.82‡	0	>25	>5.3	0
2	11/57	20	3.15	0	24.0	3.78	0
3	8/57	7.5	0.18‡	0.01	4.4	0.11	0.006
4	11/57	7.5	0.61	0.07	12.6	1.02	0.12
5	11/57	12	2.49	0	5.6	1.17	0
6	5/58	15	1.99‡	0	11.0	1.46	0
7	10/57	7.5	0.83	0	5.6	0.63	0
8	1/57	15	0.70	0	16.2	0.76	0
9	5/56	15	0.67	0.27	22.4	1.00	0.41
10	1/57	20	1.19	1.19	23.5	1.40	1.40
11	5/58	7.5	1.32‡	0	6.0	1.05	0
12	10/56	40	7.02	0	13.9	2.44	0
13	1/57	22	6.06	0	8.7	2.40	0
14	3/58	18	2.22‡	0	33.2	4.10	0
15	2/56	20	1.60	0	12.5	1.00	0
16	12/58	16	0.60	0	9.6	0.36	0
17	12/56	7.5	0.20	2.00	18.8	0.50	5.00
18	4/57	6.0	1.73‡	0	8.8	2.51	0
19	11/56	25	0.63	0	27.6	0.70	0
20	7/57	20	2.67	0	19.4	2.58	0
21	10/56	30	2.30	0	50.0	3.83	0
22	9/55	12	0.52	0	6.3	0.28	0
23	5/56	7.5	0.08	0	8.2	0.08	0
24	6/56	20	1.64	0	17.1	1.40	0
25	12/56	10	0.36‡	0.04	9.6	0.35	0.04
26		25					
27	4/56	17.5	0.67	0	20.7	0.79	0
28	10/56	30	0	1.04	54.7	0	1.90
29	10/56	30	0.74	0.37	39.8	0.98	0.49
30	2/59	20	0.14‡	0.08	27.6	0.20	0.11
Mean		17.6 (S.D. = 8.3)	1.63 (S.D. = 1.7)	0.17 (S.D. = 0.45)	18.7 (S.D. = 12.7)	1.45 (S.D. = 1.35)	0.32 (S.D. = 0.97)

* Micrograms per milligram creatinine.
† Milligrams per 24 hours.
‡ Technique of von Euler and Floding (10); all other catechol determinations done by bioassay (11).

fore interesting that the mean VMA excretions of normal and hypertensive subjects were the same in the present study. However, caution must be exercised in using this as evidence of normal NE metabolism or physiologic function in primary hypertension, since both E and NE give rise to VMA (3, 5, 7), and since the locus as well as the rate of VMA formation are not revealed by these figures. Moreover, it has not been proven that all urinary VMA is of endogenous origin.

Urinary NE excretion has been shown to diminish rapidly within two hours of its intravenous administration (13). On the other hand, Goodall and co-workers (13), found high C^{14}-labeled VMA excretion from the first to the twenty-fourth hour after giving a normal subject NE-2-C^{14} intravenously. These findings suggest that the VMA excretion of a patient with a pheochromocytoma and paroxysmal hypertension may be more consistently elevated than the NE excretion. This situation might explain the association of a normal NE excretion and an abnormal VMA excretion in the urine specimen from the patient, No. 23 (Table III). Similarly, Kraupp, Stormann, Bernheimer and Obenaus (17) described a patient with pheochromocytoma who excreted abnormally large quantities of VMA and catecholamines when paroxysmal hypertension occurred, but only large amounts of VMA when normotensive. Moreover, it has been established that increased urinary catecholamine excretion can occur in hypertensive patients in the absence of pheochromocytoma (18) as well as normal catecholamine excretion in the presence of pheochromocytoma (19). It is for these reasons as well as for the relative simplicity of urinary VMA analysis that this test is believed to represent a useful adjunct for the diagnosis of pheochromocytoma.

Such sympathomimetic drugs as ephedrine, phenylephrine, naphazoline and isopropylarterenol failed to affect VMA excretion. Isopropylarterenol does interfere with both the bioassay and fluorimetric techniques for urinary catecholamine determination. Other pharmacologic agents are known to interfere with the fluorimetric analysis of urinary catecholamines (20), but their effect upon VMA excretion was not studied.

The VMA excretions of five patients with pheochromocytomas have been reported by others (3, 5), and show good agreement with the data of this study. Our normal range of VMA excretion is in close agreement with the figures noted by Armstrong and associates (3, 9).

The minor modification of Armstrong's VMA technique (9), by increasing the ethyl acetate extractions from two to three, was required for adequate VMA recovery (over 90 per cent). Another modification, that of subjecting the urine aliquot to mild acid and heat, permitted destruction of the only substance other than VMA which caused a significant purple spot on these chromatograms (R_f 0.35 in NH_3 phase and 0.29 in benzene phase). This procedure did not change the quantitative estimate of either naturally occurring or added VMA in the urine. Not only did this modification permit more simplified reading of the chromatograms, but it also allowed formulation of a new rapid colorimetric procedure which may be used for screening patients with pheochromocytomas from those with primary hypertension (21).

It is noteworthy that the ratio of VMA to NE and E excreted by normal subjects (Table I) and those with primary hypertension (Table II) approximates 100:1, but is only 10:1 in the pheochromocytoma group. The latter ratio agrees more closely with the previously noted data (5–7, 13) derived from the parenteral administration of labeled E and NE. Apparently the body's metabolism of these substances differs at least quantitatively with the route and/or rate of their administration. Relatively rapid introduction of large quantities of E and NE into the systemic veins (as may also occur from a pheochromocytoma) appears to favor urinary excretion of the amine relative to the phenolic acid. Thus, caution must be exercised in drawing conclusions regarding the physiologic degradation of catecholamines from studies of their urinary metabolites following parenteral administration.

SUMMARY

1. A product of catecholamine metabolism, vanillylmandelic acid, has been chromatographically determined in the urine of 15 normal subjects, 36 patients with primary hypertension, and 30 patients with pheochromocytomas.

2. All patients with pheochromocytomas demonstrated abnormally large amounts of vanillylmandelic acid in their urine.

3. Normal subjects and patients with primary hypertension excreted the same mean quantity of vanillylmandelic acid. No overlapping occurred between these groups and the pheochromocytoma group.

4. This method for urinary vanillylmandelic acid determination is helpful for the diagnosis of pheochromocytoma.

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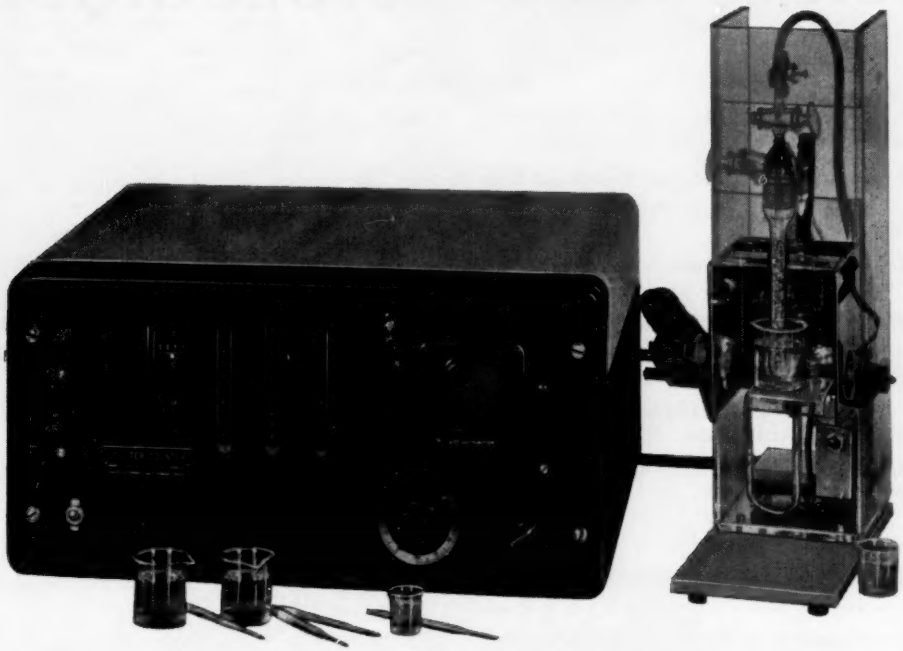

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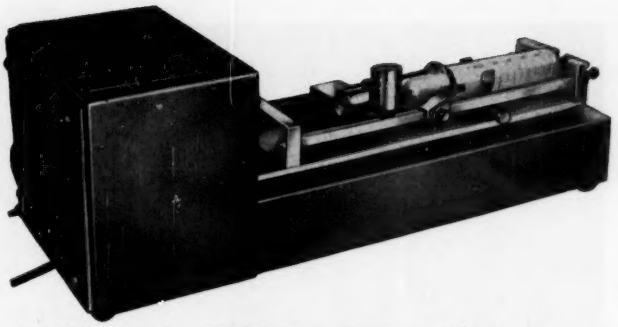

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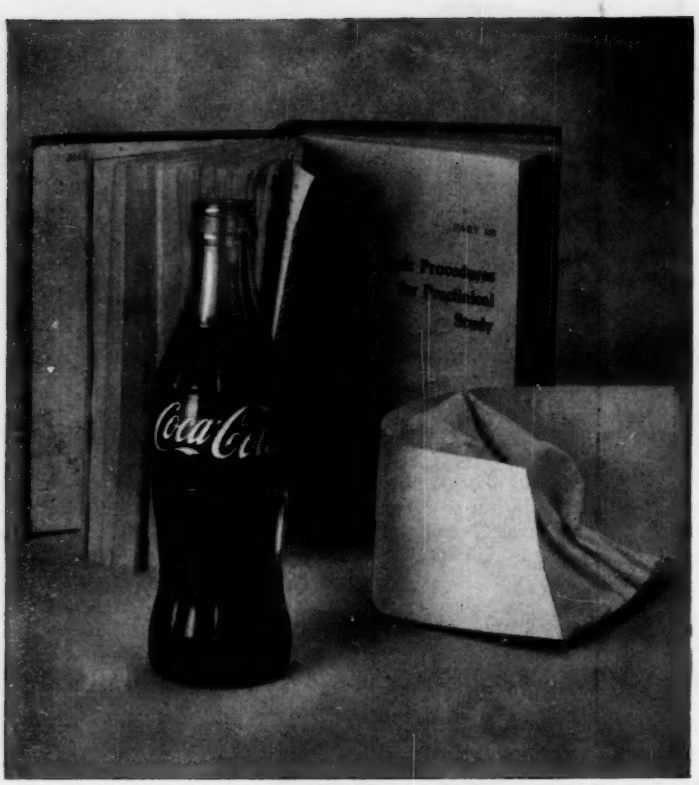

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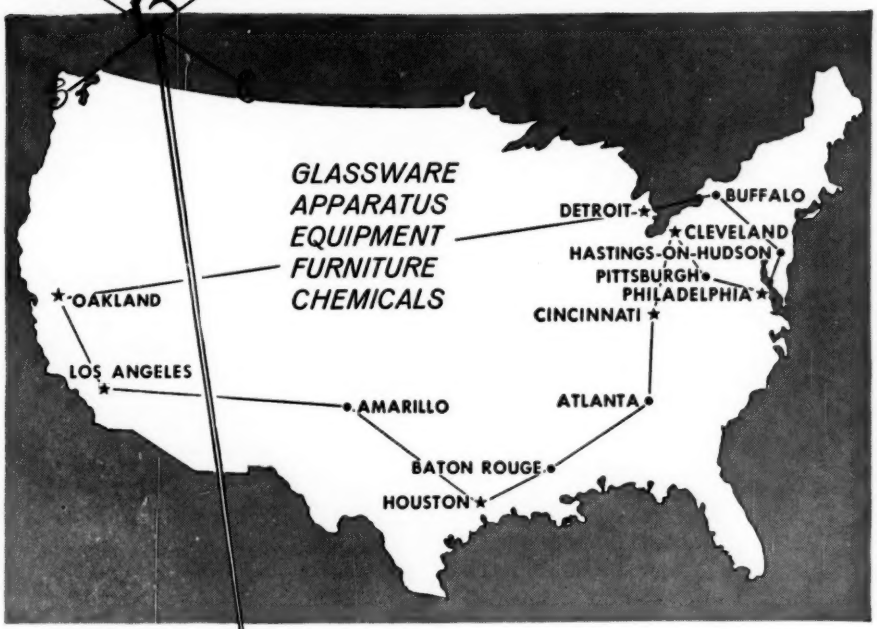

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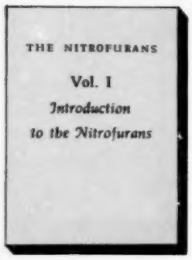
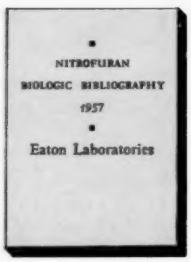


INTRODUCTION TO THE NITROFURANS

traces the development of the nitrofurans from their historical origins to their present status as clinical antimicrobial agents.

Upon request.



NITROFURAN BIOLOGIC BIBLIOGRAPHY

a compilation of all references to the nitrofurans in the medical literature; to be revised annually.

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